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The Reactivity of the Combined Cystine of Proteins other than Wool

By H. LINDLEY, *Wool Industries Research Association, Torridon, Headingley, Leeds* 6

(*Received* 21 *August* 1947)

Previous work has shown that the action of bisulphite solutions on wool leads to the subdivision of the combined cystine into two main fractions of differing chemical reactivity (Elsworth & Phillips, 1941; Middlebrook & Phillips, 1942*a*), each of which can be divided into two fractions showing smaller differences in reactivity. Further work on the reaction of wool with formaldehyde (Middlebrook & Phillips, 1942*b*, 1947) and alkalis (Cuthbertson & Phillips, 1945; Lindley & Phillips, 1945) has provided evidence that the combined cystine can be subdivided by reagents other than bisulphite. The effect of chemical modification of the wool on the magnitudes of the cystine fractions has been studied (Lindley & Phillips, 1947) and has helped to interpret the results in terms of protein structure.

In these circumstances it appeared important to determine whether the reactivity of the combined cystine varies in all cystine-containing proteins, or whether such variation is confined to the keratins or even to wool itself. The S distributions of a number of different types of keratin and of some other non-fibrous proteins have therefore been determined. The results obtained show that this variation in reactivity of the combined cystine is characteristic of the keratins and, with some slight reservations, of other cystine-containing proteins. The combined cystine of all the keratins examined has thus been found to be divisible into two main fractions: $(A+B)$ defined as combined cystine which yields thiol plus *S*-cysteinyl sulphonate groups with $NaHSO_3$ and lanthionine with dilute alkalis, and $(C+D)$ defined as being inert towards cold $NaHSO_3$ and giving combined α-aminoacrylic acid with alkalis. Fraction $(A+B)$ of these proteins has been further divided into subfraction *A* which gives water-unstable thiol and *S*-cysteinyl sulphonate groups with $NaHSO_3$, and subfraction *B*, which gives water-stable thiol and *S*-cysteinyl sulphonate groups. No attempt has been made to differentiate between subfractions *A* and *B* of the non-keratinous proteins.

THE $(A+B)$ AND $(C+D)$ FRACTIONS OF THE COMBINED CYSTINE OF VARIOUS KERATINS

Experimental

Materials. These call for no special comment beyond the fact that they were purified before analysis by successive extraction with ethanol and water, the sole exceptions to this being the cow- and sheep-horn samples which are asterisked in Tables 1–4. After these latter had been partially analyzed, it was discovered that they had previously been boiled in water for some hours to remove the non-keratinous cores. New material was accordingly analyzed, but both sets of analyses are recorded for completeness. To facilitate penetration of the $NaHSO_3$ solution, all the horn samples were converted to fine shavings in a milling machine.

Analytical methods. These are identical with those previously described (Lindley & Phillips, 1945, 1947) with the addition of amide-N estimations, which were carried out as follows: 5 ml. samples of the hydrolysates (1 g. hydrolyzed for 4 hr. with 10 ml. 5 N-HCl, filtered and made up to 50 ml.) were transferred to micro-Kjeldahl flasks and made just alkaline (bromocresol green) with 2 N-NaOH; 5 ml. of 2% (w/v) borax were added to bring the pH to 8–9, and the NH_3 removed by distillation in a micro-Kjeldahl apparatus.

Results

The S distribution of the keratins

The results of various S estimations on a variety of keratins are given in Table 1 together with the percentage of S unaccounted for either as cystine, cysteine, methionine, Br-oxidizable S or sulphate S. It will be seen that the keratins have a wide range of values for cysteine, cystine, total and unidentified S. Particularly noteworthy are the high cystine content of the dog hair and the high cysteine content of the two horn samples first analyzed. This high thiol content could not be duplicated on the other two samples by boiling them with water, and therefore this probably represents a real difference between the two specimens. The wide variation in the analytical figures of the various horn samples is of some interest.

Another point of importance is the wide range of values found for the unidentified S of the keratins. Thus the total S of dog hair, sheep horn and duck feathers can almost quantitatively be accounted for as cystine, methionine and Br-oxidizable + sulphate S, whereas calf, cow-tail and rabbit hair all contain approximately 0·8% S which remains unidentified. That this is not necessarily due to treatment of the keratin prior to analysis is established conclusively in the case of the rabbit hair. This particular specimen was obtained from the rabbit used to prepare the myosin for the later experiments and had no subsequent treatment other than extraction with ethanol and water. It also seems improbable that this unidentified S arises by oxidation of cystine

Table 1. *The S distribution of certain keratins*

(Results as % of protein dry wt.)

Keratin	Total S	Sulphate S + Br-oxidizable S	Thiol S	Disulphide S	Methionine S	Unidentified S (by diff.)
Dog hair	5·30	0·29	0·15	4·65	0·16	0·05
Rabbit hair	4·35	0·27	0·16	2·94	0·22	0·76
Calf hair	3·97	0·45	0·05	2·53	0·07	0·87
Cow-tail hair	3·93	0·28	0·05	2·71	0·10	0·79
Horse hair	3·71	0·19	0·00	2·83	0·19	0·50
Camel hair	3·28	0·09	0·00	2·74	—	—
Alpaca	3·93	0·14	0·00	3·34	0·08	0·38
Yak hair	3·85	—	0·00	2·78	—	—
Reindeer hair	—	—	0·00	2·40	—	—
Duck feathers	3·26	0·19	0·01	2·75	0·10	0·21
Cow horn*	4·17	0·37	0·54	2·37	0·08	0·81
Sheep horn*	—	0·19	0·34	2·68	—	—
Cow horn	3·77	0·07	0·22	2·94	0·14	0·40
Sheep horn	2·08	0·01	0·10	1·69	0·14	0·14
Foetal lamb's wool	3·64	0·08	0·13	2·95	—	—

* Pre-boiled in water.

Table 2. *The subdivision of the combined cystine of keratins into fractions (A + B) and (C + D)*

(Results in terms of S as % of protein dry wt.)

Keratin	Fraction (A + B)	Fraction (C + D) (by diff.)	Subfraction B	Subfraction A (by diff.)
Dog hair	2·52	2·28	0·64	1·88
Rabbit hair	1·63	1·47	0·40	1·23
Calf hair	1·13	1·45	0·35	1·10
Cow-tail hair	1·62	1·14	0·69	0·93
Horse hair	1·75	1·08	0·46	1·29
Camel hair	1·39	1·35	0·35	1·04
Alpaca	1·37	1·97	0·52	0·85
Yak hair	0·96	1·82	0·31	0·65
Reindeer hair	0·77	1·63	0·27	0·50
Duck feathers	2·04	0·72	0·86	1·18
Cow horn*	2·03	0·88	0·50	1·53
Sheep horn*	1·56	1·46	—	—
Cow horn	2·22	0·94	0·63	1·59
Sheep horn	1·20	0·59	0·34	0·86

* Pre-boiled in water.

during hydrolysis, since Baernstein estimations (in which the hydrolysis is carried out in a reducing medium throughout) gave values of 2·48, 2·72 and 3·16 % S respectively for the calf hair, cow-tail hair and rabbit wool compared with 2·58, 2·76 and 3·10 % S for the thiol + disulphide S by Shinohara estimation. Two-dimensional partition chromatography on filter paper of the hydrolysate of rabbit wool did not reveal the presence of lanthionine or any new amino-acid. A very faint spot corresponding to cysteic acid did occur, but was not of sufficient intensity to account for the unidentified S. Pending the result of further investigations, now in progress, the question of the chemical nature of this unidentified S must therefore be left open.

The subdivision of the combined cystine into subfractions A and B and fraction (C + D)

The various keratins were treated with 30 % (w/v) $NaHSO_3$ overnight, hydrolyzed and the thiol S content of the hydrolysates estimated to give fraction (A + B). Fraction (C + D) was calculated by subtracting this value from the disulphide S (Table 1). The results obtained are given in Table 2, together with the determinations of subfraction B and calculated values for subfraction A.

From Table 2 it will be seen that all the proteins contain both fractions (A + B) and (C + D) and that the magnitude of these fractions varies both absolutely and relatively. When the original

fractionation of the cystine S of wool was first reported (Middlebrook & Phillips, 1942*a*) some stress was laid on the approximate equality of fractions $(A+B)$ and $(C+D)$ of wool. The present analyses show that this is not a general property of keratins. By virtue of its very high cystine content, dog hair has the highest absolute value for both fractions $(A+B)$ and fraction $(C+D)$. The next highest absolute values for fraction $(A+B)$ are shown by duck feathers and cow horn, but if fraction $(A+B)$ is expressed as a percentage of either the total S or the total disulphide S, then duck feathers easily provide the highest value. This is of some interest since duck feathers are the only representative of the naturally occurring β-keratins here analyzed. The keratin with the lowest $(A+B)$ fraction both absolutely and relatively was reindeer hair, which was also undoubtedly the least impressive fibre of the varieties studied, being of very irregular cross-section, and with little tensile strength.

Whilst fraction $(A+B)$ does not in general form any simple fraction of either the total disulphide S or total S a rather remarkable regularity comes to light when the relative magnitudes of subfractions A and B are compared (Table 3). In six of the keratins, dog hair, cow horn, rabbit wool, calf hair, horse hair and camel hair, subfraction A is approximately three times as great as subfraction B. In two of the keratins, yak hair and reindeer hair, the ratio is 2 to 1, whilst in cow-tail hair and duck feathers it is approximately 4 to 3; the alpaca gives an isolated figure of 1·6 (3) to 1, and the cow and sheep horn each give a ratio of 2·5 to 1. The ratio of subfraction A to subfraction B in the wool analyzed by Lindley & Phillips (1947) was 2 to 1.

Lindley & Phillips (1947) pointed out that subfraction A of the combined cystine of wool appeared to be intimately associated with the free carboxyl groups, since esterification of these carboxyl groups altered the cystine reactivity so that this subfraction behaved like subfraction B. A not unreasonable assumption, therefore, is that subfraction B is associated with amide groups of the wool. Accordingly the amide-N contents of the keratins were determined, Table 3, where it is seen that no obvious correlation exists between the magnitude of subfraction B of the various keratins and their amide-N contents.

Table 3. *Ratio between fraction* $(A+B)$ *and total and disulphide* S, *and between subfractions A and B, together with amide-*N *values*

Keratin	$(A+B)$/Total S	$(A+B)$/Disulphide S	A/B	Amide N (% of dry wt.)
Dog hair	0·48	0·53	2·9	1·11
Rabbit wool	0·37	0·53	3·1	1·07
Calf hair	0·28	0·44	3·1	1·20
Cow-tail hair	0·31	0·59	1·35	1·26
Horse hair	0·41	0·62	2·8	1·22
Camel hair	0·42	0·51	3·0	1·25
Alpaca	0·35	0·41	1·6	1·27
Yak hair	0·25	0·35	2·1	1·34
Reindeer hair	—	0·32	1·9	1·39
Duck feathers	0·62	0·74	1·4	1·31
Cow horn*	0·49	0·70	3·1	1·24
Sheep horn*	—	0·52	—	—
Cow horn	0·59	0·70	2·5	1·14
Sheep horn	0·58	0·67	2·5	1·20

* Pre-boiled in water.

THE $(A+B)$ AND $(C+D)$ FRACTIONS OF THE COMBINED CYSTINE OF SOME NON-KERATINOUS PROTEINS

Experimental

Materials. (1) Egg albumin: A commercial sample from British Drug Houses Ltd. and also a crystalline preparation made by the procedure of Kekwick & Cannan (1936). (2) Gliadin: This and the two succeeding proteins were gifts from Prof. A. C. Chibnall. (3) Edestin: Recrystallized. (4) Ground nut globulin. (5) Rabbit myosin: Prepared from the leg muscle of the rabbit according to the method of Greenstein & Edsall (1940). (6) Insulin: British Drug Houses Ltd., crystalline. (7) Lactoglobulin: This was prepared according to Palmer (1934), but could not be induced to crystallize and was eventually precipitated from solution by the addition of ethanol. (8) Serum albumin: A crude commercial preparation (British Drug Houses Ltd.).

Treatment with $NaHSO_3$. The protein (*c.* 0·5 g.) was treated with $NaHSO_3$ (1 g.) and water (3 ml.) overnight. Conc. HCl (5 ml.) was then added and the mixture boiled under reflux for 4 hr. Control experiments have shown that this method can be used to determine fraction $(A+B)$ satisfactorily for wool.

Results

Tables 4 and 5 give the results of the analyses together with those previously reported by other investigators. The results were calculated on a dry weight basis calculated from the N content (Kjeldahl) of the hydrolysates. From these results it is apparent

Table 4. *The thiol and disulphide* S *content of protein hydrolysates*

(Results in terms of S as % of protein dry wt.)

	Experimental results			Other investigators			
Protein*	SH	SS	SH + SS	SH	SS	SH + SS	Reference
Egg albumin (commercial) (15·8% N)	0·07	0·45	0·52	—	—	—	—
Egg albumin (crystalline) (15·8% N)	—	—	—	0·37	0·28	0·65	Hess & Sullivan (1943)
	0·17	0·12	0·29	—	—	0·48	Kassell & Brand (1938)
Myosin (16·8% N)	0·0	0·19	0·19	0·29	0·04	0·33	Hess & Sullivan (1943)
	—	—	—	—	—	0·21	Bailey (1937*a*)
Gliadin (17·4% N)	0·01	0·48	0·49	—	—	0·64, 0·69	Bailey (1937*b*)
Edestin (18·6% N)	0·0	0·32	0·32	0·09	0·26	0·35	Hess & Sullivan (1943)
	—	—	—	—	—	0·38	Lugg (1938)
Lactoglobulin (15·6% N)	0·11	0·71	0·82	0·29	0·61	0·90	Brand & Kassell (1942)
Insulin (15·5% N)	0·0	2·60	2·60	0·0	3·32	3·32	Miller & du Vigneaud (1937)
Insulin HCl-HCOOH	0·0	2·96	2·96	—	—	—	—
Serum albumin (15·7% N)	0·0	1·25	1·25	0·08	1·57	1·65	Hess & Sullivan (1943)
Ground nut globulin (18·3% N)	0·02	0·20	0·22	—	—	—	—

* The N content used in the calculation of protein wt. is given in parenthesis.

Table 5. *Fraction* $(A + B)$ *of the combined cystine of various proteins*

(Results in terms of S as % of protein dry wt.)

Protein	SH of fraction $(A + B)$	SS + SH	Maximum SH in protein	Reference
Egg albumin (commercial)	0·30	0·53	—	—
Egg albumin (crystalline)	0·15	0·36	0·34, 0·26	Greenstein (1938), Mirsky (1941)
Myosin	0·08	0·28	0·31	Greenstein & Edsall (1940)
Gliadin	0·26	0·56	—	—
Edestin	0·18	0·31	0·13	Greenstein (1939)
Lactoglobulin	0·38	0·81	—	—
Insulin	0·72	2·56	0·0	Greenstein (1944)
Serum albumin	0·70	1·08	0·09	Greenstein (1940)
Ground nut globulin	0·06	0·23	—	—

that the combined cystine of non-keratinous proteins can also be divided into fractions which are reactive and unreactive to $NaHSO_3$ solutions at pH 5 and room temperature. In most of these cases the $(A + B)$ fraction is approximately half of the total disulphide, the principal exceptions being myosin and insulin, and with both these complications result from losses of S during hydrolysis. The confirmation of the finding of Miller & du Vigneaud (1937) that increased recovery of cystine from insulin can be obtained by hydrolyzing in the presence of formic acid is of interest since unpublished work by Mr W. R. Middlebrook in this laboratory has shown that this technique of hydrolysis does not affect the recovery of cystine from wool. Although the present value for the cystine content of insulin is lower than that of Miller & du Vigneaud it is in agreement with the work of Sahyun (1941) who prepared crystalline insulin with 2·96% total S. The present value has also been checked by Baernstein's procedure which gave 2·90% S as cystine + cysteine.

DISCUSSION

It has long been realized that the sulphur and cystine contents of keratins vary widely and that this variation occurs even within one type of keratin, e.g. wool. It seemed possible, however, that some regularities might be revealed by considering the differing cystine fractions separately, but the results obtained in the present work lend little support to this view. A possible exception to this is that subfraction *A* may be a simple multiple of subfraction *B*, but even this doubtful relationship is not constant throughout the keratins.

The observation that the combined cystine of the other proteins examined also shows differing reactivity towards $NaHSO_3$ solutions is important, since it suggests that the ultimate cause of this effect will be found in some relatively simple factor of protein structure. These results also make it extremely improbable that the differing reactivity

of the various fractions of the combined cystine of wool are directly due to the heterogeneous nature of wool. It was also considered possible that the $(A + B)$ fraction of the non-keratinous proteins might be identical with the maximum titratable thiol groups found for these proteins in solution, but it cannot be claimed that the results lend much support to this suggestion. On this point the results are in conflict with those of Hess & Sullivan (1943) who found the cysteine content of the hydrolysates of a number of proteins to be equivalent to the titratable thiol groups of the intact protein. Unfortunately few independent estimations are available, but it would seem that in the case of myosin the present results are in agreement with the findings of Bailey (1937 *a*, *b*).

SUMMARY

1. It has been shown that the combined cystine of a number of keratins can be subdivided into fractions of differing reactivity towards $NaHSO_3$ solutions in a manner analogous to that previously found for the combined cystine of wool.

2. Evidence of a similar subdivision of the combined cystine S has been found for other non-fibrous proteins.

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REFERENCES

Bailey, K. (1937*a*). *Biochem. J.* **31**, 1396.
Bailey, K. (1937*b*). *Biochem. J.* **31**, 1406.
Brand, E. & Kassell, B. (1942). *J. biol. Chem.* **145**, 365.
Cuthbertson, W. R. & Phillips, H. (1945). *Biochem. J.* **39**, 7.
Elsworth, F. F. & Phillips, H. (1941). *Biochem. J.* **35**, 135.
Greenstein, J. P. (1938). *J. biol. Chem.* **125**, 501.
Greenstein, J. P. (1939). *J. biol. Chem.* **128**, 233.
Greenstein, J. P. (1940). *J. biol. Chem.* **136**, 795.
Greenstein, J. P. (1944). *Chem. Rev.* **34**, 157.
Greenstein, J. P. & Edsall, J. T. (1940). *J. biol. Chem.* **133**, 397.
Hess, W. C. & Sullivan, M. X. (1943). *J. biol. Chem.* **151**, 635.
Kassell, B. & Brand, E. (1938). *J. biol. Chem.* **125**, 145.
Kekwick, R. A. & Cannan, R. K. (1936). *Biochem. J.* **30**, 227.
Lindley, H. & Phillips, H. (1945). *Biochem. J.* **39**, 17.
Lindley, H. & Phillips, H. (1947). *Biochem. J.* **41**, 34.
Lugg, J. W. H. (1938). *Biochem. J.* **32**, 2123.
Middlebrook, W. R. & Phillips, H. (1942*a*). *Biochem. J.* **36**, 428.
Middlebrook, W. R. & Phillips, H. (1942*b*). *Biochem. J.* **36**, 294.
Middlebrook, W. R. & Phillips, H. (1947). *Biochem. J.* **41**, 218.
Miller, G. L. & du Vigneaud, V. (1937). *J. biol. Chem.* **118**, 101.
Mirsky, A. E. (1941). *J. gen. Physiol.* **24**, 709.
Palmer, A. H. (1934). *J. biol. Chem.* **104**, 359.
Sahyun, M. (1941). *J. biol. Chem.* **138**, 487.

Preparation and Antibacterial Action of some Compounds Structurally Related to Glutamic Acid. Their Application in Microbiological Determination of Small Quantities of Glutamine

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In studying the metabolism of glutamine (McIlwain, Roper & Hughes, 1948) use was made of compounds structurally related to glutamic acid which differentially inhibited certain of the reactions concerned. These inhibitors were selected from a larger group of analogues and derivatives of glutamic acid, whose preparation and properties are described in the present paper.

The substances concerned are listed in Table 1. Expectation that γ-glutamylhydrazine might be inhibitory was based on analogous findings with respect to pantoylhydrazine (Madinaveitia, Martin, Rosé & Swain, 1945; McIlwain & Hughes, 1945). Inhibition by α-amino-γ-methylsulphinylbutyric acid or 'methionine sulphoxide' was reported while the present investigations were in progress (Borek, Miller, Scheiness & Waelsch, 1946; Waelsch, Owades, Miller & Borek, 1946). Several examples are known of inhibition by homologues of biologically active compounds and this led to the examination of the α-amino dicarboxylic acids of Table 1.

Table 1. *Effects on streptococcal growth, glycolysis and glutamine hydrolysis, of compounds structurally related to glutamine*

((*a*) The growth response of *Streptococcus faecalis* (laboratory strain, *F*) was examined in the medium employed in assay, but lacking the yeast preparation and methionine sulphoxide; for the Richards strain (*R*), glutamine (final concentration, 5×10^{-5} M) was added. Substances were examined at the following concentrations: 2×10^{-2} M, 4×10^{-3} M and 8×10^{-4} M, except in the cases of compounds (1), (3) and (6) when the highest concentration was 10^{-2} M. No compound replaced glutamine in permitting growth of the glutamine-requiring streptococci nor in stimulating streptococcal glycolysis. γ-Ethyl glutamate behaved towards the exacting streptococci in a fashion similar to glutamic acid, in that high concentrations permitted a delayed growth in absence of glutamine. This did not occur with the other compounds examined, and was presumably due to glutamic acid formed from the ester.

(*b*) Glycolysis was carried out anaerobically in $NaHCO_3$-NaCl solution (cf. McIlwain, 1946). Compounds were examined at the following concentrations: (2), (4), (5), (7), (9) and (10): 0·033, 0·0067, and in some cases 0·0013 M; of (1), (3), (6), (8) 0·016 and 0·0016 M.)

Compound	(*a*) Effect on growth: Inhibition (+) or absence of inhibition (0)	(*a*) Effect on growth: Organisms examined	(*b*) Effect on glycolysis or NH_3 formation from glutamine: Inhibition (+) or absence of inhibition (0) not tested (−)	(*b*) Effect on glycolysis or NH_3 formation from glutamine: Organisms examined
I. α-Amino dicarboxylic acids				
(1) α-Amino-α-propylsuccinic acid	0	*R*	0	*R*
(2) α-Amino-β-butylsuccinic acid	0	*R*	0	*R*
(3) α-Amino-β-octylsuccinic acid	0	*R*	0	*R*
(4) α-Aminoadipic acid	0	*R*	0	*R*
(5) α-Aminopimelic acid	0	*R*	0	*R*
(6) α-Aminoundecane-αλ-dicarboxylic acid	0	*R*	0	*R*
II. Other amino carboxylic acids				
(7) α-Amino-α-methylvaleric acid	0	*R*	0	*R*
(8) α-Amino-β-furylpropionic acid	0	*R*	0	*R*
III. Methionine derivatives				
(9) α-Amino-γ-methylsulphinylbutyric acid	0 +	*R* *F*	0	*R, F*
(10) α-Amino-γ-methylsulphonylbutyric acid	0	*R, F*	−	
IV. Glutamic acid derivatives				
(11) *N*-(γ-Glutamyl)hydroxylamine	+	*R, F*	−	−
(12) γ-Glutamylhydrazine	+	*R, F*	+	*R*
(13) Acetone derivative of (12)	+	*R, F*	−	−
(14) γ-Ethyl glutamate	0	*R, F*	−	−

Knowledge that one of the analogues, namely methionine sulphoxide, inhibited the synthesis of glutamine from glutamic acid suggested its application in the microbiological determination of glutamine. A method for determining 10–20 mμmol. of glutamine was required in studying the metabolism of the compound. The existing methods, either chemical or enzymic (cf. Archibald, 1945) depend finally on determination of NH_3 produced from glutamine under conditions more or less specific for glutamine. If the NH_3 was estimated by photometric measurement of the colour yielded with Nessler's reagent, quantities of about 500 mμmol. of NH_3 could be determined in this way. Such methods were, however, not necessarily sensitive to 500 mμmol. of glutamine. Thus it was necessary that any NH_3 initially present, or produced non-specifically under the conditions of hydrolysis of glutamine, should be removed or determined separately by blank estimations. In many cases we desired to determine glutamine in the presence of up to 100 times its (molar) quantity of ammonium glutamate. This demanded a very complete removal or accurate estimation of the NH_3, and in practice the determination of glutamine by the NH_3 formed from it did not give satisfactory results.

The possible use of microbiological methods for determining glutamine presents problems of a different type. The growth response of virulent group A β-haemolytic streptococci was applied in detecting and roughly estimating glutamine in natural materials in 1939 (McIlwain, Fildes, Gladstone & Knight, 1939). Since that time glutamine has been described as stimulating the growth of other micro-organisms (cf. Knight, 1945) and in some cases an organism's response to glutamine has been studied in detail as a possible basis for assay of glutamic acid or glutamine. In all such cases, difficulty has been encountered in discriminating between the effects of glutamic acid, ammonium salts,

and glutamine. Response to glutamic acid has been found slower than response to glutamine with both streptococci (McIlwain *et al.* 1939) and *Lactobacillus arabinosus* (Lewis & Olcott, 1944; Lyman, Kuiken, Blotter & Hale, 1945; Hac, Snell & Williams, 1945); and equimolar concentrations of the substances may finally give the same response, or glutamine may give the greater response. Hac *et al.* (1945) applied the different chemical properties of glutamic acid and glutamine, together with their effects on the growth of *L. arabinosus*, to the determination of glutamine.

In the present study it was preferred to try to obtain conditions of growth which more sharply discriminated between glutamic acid and glutamine. This was especially necessary in determining glutamine in the presence of a large excess of glutamic acid. Conditions were therefore chosen in which ammonium and glutamic acid salts were throughout present in a deficient basal medium consisting of glucose-peptone with various addenda. Recently passaged β-haemolytic streptococci did not grow in this medium within 5 days or so unless glutamine was added. With excess glutamine, maximum growth was usually reached in 18–24 hr. Sub-optimal concentrations of glutamine resulted in less growth, but although the graded response of Fig. 1*A* was obtained, this was not a suitable basis for the assay of glutamine. The reason for this can be seen by considering the wide differences between the curves obtained from readings at different periods of growth. With quantities of glutamine between 5 and 25 mμmol., readings increase 2- to 3-fold between 18 and 25 hr.; at 5 or 10 mμmol. readings increase 2- to 6-fold between 25 and 41 hr. Any substance, therefore, which was present in a material being assayed for glutamine, and which accelerated or retarded growth of the streptococci during assay, would cause large variations in the organism's response to a given quantity of glutamine.

Growth of the type shown in Fig. 1*A* appeared likely to be due to synthesis of glutamine by the organisms from ammonium glutamate. We therefore tried to obtain a more stable response to glutamine by adding α-amino-γ-methylsulphinylbutyric acid to the deficient basal medium. The glutamic acid content of this medium was about 10^{-3}M, and it was anticipated from the results of McIlwain *et al.* (1948) that, with the specimen of sulphoxide employed (a mixture of isomerides), a concentration of about 2×10^{-2}M would be required. Trial showed that this concentration offered no advantage over one of $1{\cdot}1 \times 10^{-2}$M, which was that adopted in the method as described below. Media containing the sulphoxide afforded a much more stable response to glutamine. This is illustrated in Fig. 1*B*, which shows the increase in readings between 16 and 24 hr. to be no more than 25% of their values at the earlier time. Three strains of β-haemolytic streptococci gave results similar to that of Fig. 1*B*, and of them the Richards strain was adopted for use in assay, as its response to glutamine is known to be highly specific (McIlwain, 1939).

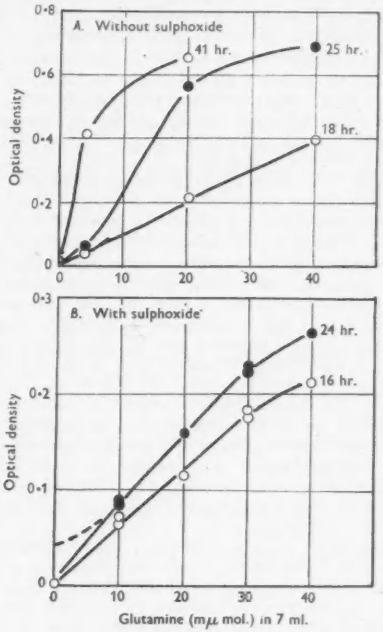

Fig. 1. Growth of streptococcal cultures in media initially containing varying quantities of glutamine, measured by their optical densities at different times. *B*, using the medium described in the text and normally employed in assay; *A*, using a medium without methionine sulphoxide, but otherwise identical with that of *B*.

EXPERIMENTAL AND RESULTS

Preparation of compounds related to glutamic acid

γ-Ethyl glutamate was prepared by the method of Bergmann & Zervas (1933) modified as follows. After esterification of glutamic acid (5 g.) in anhydrous ethanol (35 ml.) the volume was made up to 150 ml. with ethanol and British Drug Houses Ltd. Universal Indicator added. Aqueous NH_3 (sp.gr. 0·88) was added dropwise until the solution was orange-red, when the precipitated NH_4Cl was removed at the pump. The filtrate was cautiously made more alkaline with NH_3, any immediate amorphous precipitate being removed by filtration. The solution was finally brought to an orange-yellow colour, when the ester separated as plates. The whole was kept several hours at 0° and filtered, the filtrate being tested with NH_3 for a further precipitation of ester. After recrystallization from aqueous ethanol yields of more than 90% were obtained.

γ-Glutamylhydrazine. γ-Ethyl glutamate (5 g.) with 50% (w/w) hydrazine hydrate (10 ml.) and water (5 ml.) were warmed at 70° for 15 min. Absolute ethanol (200 ml.) at 70° was added slowly and the mixture kept at 0° for several hours. The fine crystalline precipitate was filtered off and recrystallized from aqueous ethanol. A white crystalline solid (3·9 g., yield 72%; m.p. 165°) was obtained. (Melting points here and elsewhere are uncorrected.) (Found: C, 32·2, 31·5; H, 7·38, 7·42; N, 22·2%; loss in weight at 105°, 16·3%. $C_5H_{11}O_3N_3.1{\cdot}5H_2O$ requires C, 31·9; H, 7·44; N, 22·3; H_2O, 14·6%.) To minimize the possibility of glutamine production from γ-ethyl glutamate and NH_3 present as impurity in the hydrazine hydrate, the purest specimen of hydrazine hydrate available was used. This was estimated to contain 0·14 mol. % of NH_3.

Picrate. γ-Glutamylhydrazine (260 mg.) with picric acid (360 mg.) and water (30 ml.) was heated until complete solution was obtained. A yellow solid separated on cooling. This was filtered off and crystallized three times from water containing 5% (v/v) of ethanol. A fine yellow crystalline solid (445 mg., yield 78%; m.p. 190° (decomposition)) was obtained. (Found: C, 33·9; H, 3·48; N, 21·5%. $C_5H_{11}O_3N_3.C_6H_3O_7N_3$ requires C, 33·9; H, 3·59; N, 21·5%.)

Acetone γ-glutamylhydrazone. γ-Glutamylhydrazine (0·29 g.) with acetone (2 ml.), ethanol (4 ml.) and water (1 ml.) was warmed on a water bath for 10 min. The clear solution obtained was kept at 0° for several hours. Acetone (50 ml.) was added. A microcrystalline solid separated and was filtered, washed with small portions of ice-cold ethanol and recrystallized from aqueous acetone, giving a white microcrystalline solid (150 mg.; yield, 70%; m.p. 171°). (Found: H, 7·58, 7·40, 7·65; N, 21·2%. $C_8H_{15}O_3N_3$ requires H, 7·46; N, 20·9%.)

Benzaldehyde γ-glutamylhydrazone. γ-Glutamylhydrazine (1 g.) with benzaldehyde (2 ml.) and ethanol (25 ml.) was heated on a water bath for 10 min. Water (25 ml.) was added gradually and a clear solution was obtained on continued heating. The solution was cooled slowly to 0° and held there for several hours. The white solid which slowly precipitated was filtered off and recrystallized from aqueous ethanol to give an odourless crystalline solid (1·06 g., yield 80%; m.p. 192° with decomposition). (Found: C, 57·5; H, 6·08%. $C_{12}H_{15}O_3N_3$ requires C, 57·8; H, 6·02%.)

β-Aspartylhydrazine. Asparagine monohydrate (10 g.) was refluxed with 50% (w/w) hydrazine hydrate (30 ml.) for 20 min. The solution was evaporated *in vacuo* to a gum which was taken up in water (15 ml.). Ethanol (70 ml.) was added gradually, the precipitated amorphous white solid filtered off, washed well with ethanol and crystallized from aqueous ethanol. It gave fine white crystals (9·0 g.; yield 92%; m.p. 194–195° with decomposition). (Found: C, 32·8; H, 6·26; N, 28·7%. $C_4H_9O_3N_3$ requires C, 32·7; H, 6·12; N, 28·8%.)

N-(γ-Glutamyl)hydroxylamine. To hydroxylamine hydrochloride (5 g.) suspended in ethanol (20 ml.), 10N-NaOH was added slowly and with shaking until the pH became 8 to 8·5. The mixture was filtered and the filtrate (20 ml.) with γ-ethyl glutamate (1·2 g.) was kept at room temperature in a closed vessel for 16 hr. and then at 0° for 48 hr., with shaking at intervals. The clear solution was then evaporated in a vacuum desiccator over P_2O_5 for 4 days, solid matter being removed by filtration each day. On the fifth day the volume of liquid was *c.* 3 ml. The pH was adjusted with concentrated HCl to 3·2 and the solution evaporated to a gum. The gum was rubbed with ethanol (40 ml.) and left at 0° overnight. The ethanol was decanted and the semi-solid mass taken up in water (10 ml.) and slowly precipitated as a pale-brown solid by the gradual addition of ethanol (50 ml.). Three recrystallizations from aqueous ethanol yielded pale buff-coloured needles (0·64 g., yield 57%; m.p. 155°), which gave a cherry-red colour with $FeCl_3$. (Found: C, 36·6; H, 6·11; N, 17·2%. $C_5H_{10}O_4N_2$ requires C, 37·0; H, 6·17; N, 17·3%.)

N-(β-Aspartyl)hydroxylamine. Asparagine monohydrate (5 g.) and hydroxylamine sulphate (5 g.) were boiled for 20 min. in water (25 ml.). The solution was cooled and a few drops of 10% (v/v) acetic acid added. Excess of saturated aqueous copper acetate was added gradually and the dark green precipitate filtered off and washed with water (200 ml.). The yield of copper salt was almost theoretical. The salt was suspended in 50% (v/v) aqueous ethanol and 1 drop of 0·1N-H_2SO_4 added. H_2S was passed in and the mixture filtered at 60°. The pale-blue filtrate was repeatedly treated with H_2S until a clear colourless filtrate was obtained. The filtrate was evaporated to *c.* 5 ml. *in vacuo* at less than 50°. Ethanol (20 ml.) was added and the pale-brown precipitate was recrystallized three times from aqueous ethanol to yield off-white crystals (2·2 g., yield 45%; m.p. 179–180° with decomposition) which gave a cherry-red colour with $FeCl_3$. (Found: C, 32·1; H, 5·4; N, 19·2%. $C_4H_8O_4N_2$ requires C, 32·4; H, 5·2; N, 18·9%.) Estimation of the hydroxamic acid grouping by the method described below gave 97·5±3% of the theoretical value.

Determination of hydroxamic acid grouping by $KBrO_3$. The method is based on that of Junell (1934) and consists of the oxidation of the hydroxamic acid grouping with $KBrO_3$, excess bromate being estimated by its oxidation of KI with subsequent estimation of liberated I_2. Solutions are required of $KBrO_3$ (0·04M), $Na_2S_2O_3$ (0·025M), HCl (N), KI (saturated aqueous), and of the hydroxamic acid (about 0·01N). To the solution of hydroxamic acid (5 ml.) was added $KBrO_3$ (3 ml.) and N-HCl (2 ml.). Within 2 min. of the acidification excess KI solution was added and the liberated I_2 was estimated immediately with $Na_2S_2O_3$. The method gave consistent results to within 3% of the theoretical values. The time required for oxidation varied according to the hydroxamic acid being estimated. In the case of the two aminohydroxamic acids described above the oxidation was found (by spot testing with $FeCl_3$) to be complete within 1 min. under the conditions cited. For consistent results it was necessary to complete the estimation in less than 5 min. after acidification. Other hydroxamic acids (e.g. acethydroxamic acid) require up to 30 min. for complete oxidation.

α-Amino-γ-methylsulphinylbutyric acid. The method described is a modification of that used by Toennies & Kolb (1939).

DL-Methionine (10 g.) with glacial acetic acid (40 ml.) was warmed to 60° and then cooled to room temperature. Hydrogen peroxide (11 ml. of 100 vol.) was added slowly, the vessel being shaken and cooled between additions. The clear solution obtained was cooled to 0° and absolute ethanol (350 ml.) added. The mixture was cooled at 0° for several hours and the solid filtered off. The white amorphous powder (yield, 98%; m.p. 237° with decomposition) was washed with ethanol and dried *in vacuo*. Recrystallization from aqueous ethanol gave a white odourless crystalline solid (yield, 95%; m.p. 240° with decomposition). (Found:

C, 36·4; H, 6·63; N, 8·30; S, 19·4%. Calc. for $C_5H_{11}O_2NS$: C, 36·5; H, 6·68; N, 8·48; S, 19·4%.)

No attempt was made to separate the isomerides which are presumably present in this product, and which have been examined by Waelsch & Borek (1947).

α-Amino-γ-methylsulphonylbutyric acid. Preparation of this compound has been described by Toennies & Kolb (1941). The method below gives considerably poorer yield but was employed because of its simplicity.

DL-Methionine (4 g.) was suspended in 50% (v/v) acetic acid (20 ml.). $KMnO_4$ (45 ml. of 0·67 M in 50% (v/v) acetic acid) was added slowly and the solution was allowed to stand 15 min. at room temperature. H_2O_2 (100 vol.) was added dropwise until a clear solution was obtained. The straw-coloured solution was treated with charcoal, filtered, and ethanol (150 ml.) was added. The white precipitate was filtered off and recrystallized from aqueous ethanol. A white odourless crystalline solid (2·4 g., yield 53%; m.p. 254° with decomposition) was obtained. (Found: C, 33·3; H, 6·23; N, 7·6; S, 18·1, 17·5%. Calc. for $C_5H_{11}O_4NS$: C, 33·2; H, 6·08; N, 7·7; S, 17·7%.)

Compounds 1 *to* 8 *of Table* 1. These were all DL mixtures for which we are indebted to Dr J. Elks of Glaxo Laboratories Research Division.

Some microbiological properties of the compounds related to glutamic acid

The properties of the compounds related to glutamic acid have been investigated primarily in relation to haemolytic streptococci with results which are summarized in Table 1. Homologues of glutamic acid (compounds 1–6) and some other amino carboxylic acids (compounds 7 and 8) gave no instances of inhibition, of growth, of glutamine decomposition, or of glycolysis.

The two methionine derivatives proved inhibitory (see above) and the action of the *sulphoxide* (compound 9) on streptococcal growth is described by McIlwain *et al.* (1948). The *sulphone* (compound 10) had an entirely different action. Though without action on three streptococcal strains of varying glutamine requirement and also without action on a strain of *Staphylococcus aureus*, it inhibited growth of *Escherichia coli* in the medium based on ammonium lactate and described by Fildes (1938). The sulphone (0·02 M) prevented visible growth from an inoculum of *c.* 5×10^4 cells/5 ml. during the first 2 days and resulted in less growth than in controls during the following 2 days. These effects were prevented by the presence of 0·01 M-methionine and diminished by 0·002 M-methionine.

Hydrazine derivatives. The effect on streptococcal growth of the following compounds has been examined: γ-glutamylhydrazine and its acetone derivative; β-aspartylhydrazine, and hydrazine itself. Of these, the most inhibitory were hydrazine and γ-glutamylhydrazine. Hydrazine itself prevented growth of the Richards strain at 4×10^{-3} M and of the *faecalis* strain at 2×10^{-2} M, the concentrations in each case being independent of added glutamine. Inhibition by the glutamyl derivatives was, on the other hand, markedly influenced by the concentration of glutamine also present in growth media; the action of γ-glutamylhydrazine is described fully by McIlwain *et al.* (1948). Its acetone derivative acted similarly, but higher concentrations of it were required. Of the acetone derivative, 0·013 M (but not 0·01 M), prevented growth of the Richards strain for 3 days in the presence of 2×10^{-4} M-glutamine; with 2×10^{-5} M-glutamine, 5×10^{-3} M (not 3×10^{-3} M) was adequate. The benzaldehyde derivative was only slightly soluble, and at the concentrations which could be employed it was without action on growth. β-Aspartylhydrazine was required in concentrations of 4×10^{-2} M for inhibition of either the Richards or *faecalis* strains for more than 1 day, and in some cases this inhibition was diminished by either asparagine (0·01 M) or glutamine (2×10^{-4} M). Although the compound was not sufficiently active to investigate in detail, it indicates that the substitution of hydrazine by a residue similar to the glutamyl residue (as well as by the glutamyl residue itself) can produce a compound disturbing the action of glutamine. A less closely related compound, pantoylhydrazine (McIlwain & Hughes, 1945), has, it will be noted, a distinct type of action.

Hydroxylamine and hydroxamic acids. Growth of the Richards organism was partly inhibited by 4×10^{-5} M-*N*-(γ-glutamyl)hydroxylamine, and completely by concentrations of 2×10^{-4} M or greater, in the presence of either 2×10^{-6} or 2×10^{-4} M-glutamine. A *faecalis* and a group G strain were less sensitive to the compound, of which 2×10^{-3} M gave only a slight action, but this effect also was not altered by addition of glutamine. Hydroxylamine itself was inhibitory to streptococcal growth under the present conditions in concentrations of 5×10^{-5} M or more. To explain inhibition by the glutamyl derivative as due to hydroxylamine would require a considerable degree of breakdown, which model experiments showed should be detectable by reduction of Fehling's solution at room temperature. Such reduction was not found to be brought about by solutions of the glutamyl derivative; any hydroxylamine produced under the conditions of testing was found to represent only 0·01 mol. % of the glutamyl derivative used.

N-(β-Aspartyl)hydroxylamine was required in concentrations of 5×10^{-3} to 2×10^{-2} M, and *N*-acetylhydroxylamine in concentrations of 2×10^{-2} M or higher, for any inhibition of streptococcal growth. It was observed that the hydroxylamine derivatives reacted with iron salts of the growth medium to yield a yellow coloration (red in more concentrated solutions). Increase in iron content of the medium during growth did not however prevent the action of the inhibitors.

The determination of glutamine

Organisms. The Richards strain of β-haemolytic streptococci (National Collection of Type Cultures, no. 5631), passaged frequently through mice and of M.L.D. (in mice) of less than 100 cocci, was initially employed. Face masks were worn during the pipetting involved in photometric measurement of the growth of this organism. For the assay using methionine sulphoxide, a highly virulent organism was not required and this precaution was not taken. The same strain, after maintenance in laboratory media, and having a M.L.D. in mice of some 10^8 organisms, was then used. It was preserved by growing in serum broth for 7 hr., and drying *in vacuo* in a number of ampoules (cf. Hale & McIlwain, 1947). For use, a dried inoculum was grown in serum broth and from this, serum-broth-agar slopes were prepared daily for inoculation of the assay medium. Slopes were grown for 16 hr. and used only on the day following their inoculation; serum-broth cultures were kept in a refrigerator after growth and used only during the next 14 days. Incubation throughout was aerobic and at 37°. During preliminary studies, three other strains of β-haemolytic streptococci were examined as potential test organisms, but these offered no advantages over the Richards strain. As the specificity of this organism's response to glutamine in growth is already established (McIlwain, 1939), it has been used in the present study.

Medium and inoculation. A sterile solution of peptone, NaCl, KH_2PO_4 and NaOH was prepared according to McIlwain *et al.* (1939) and kept in a dark cupboard for periods of up to 1 month. For use, the following sterile solutions were added to each 100 ml. of peptone solution: the group A addenda of McIlwain (1944), 10 ml.; 0·2 M-methionine sulphoxide, 10 ml.; the yeast preparation of McIlwain (1944), 2 ml.; and N-NaOH, 2 ml. The medium was then inoculated with cocci taken from a serum-broth-agar slope; these were suspended in about 1 ml. of the peptone solution and the density of the suspension adjusted so that (by comparison with opacity standards) it contained approximately 10^8 organisms/ml. It was then diluted 1000-fold in the peptone solution and 2·5 ml. of the dilution added as inoculum. The inoculated medium was distributed in 5 ml. portions in a number of $6 \times \frac{3}{4}$ in. test tubes.

The inoculum chosen was sufficiently small not to require washing to free it from glutamine; a 5-fold increase or decrease in its size was tolerated without much effect on the assay.

Solutions containing glutamine, and incubation. 0·01 M-Glutamine was prepared at weekly intervals from the solid, sterilized by filtration through Ford's Sterimats in an apparatus of the type described by Knight & Fildes (1936) and kept cold. On the day of its use in assay, solutions were prepared from it which were 5×10^{-6}, 10^{-5}, $1{\cdot}5 \times 10^{-5}$, 2×10^{-5} and $2{\cdot}5 \times 10^{-5}$ M with respect to glutamine. Solutions to be assayed for glutamine were also filtered to sterilize them and diluted so that two or three dilutions could be expected to be within that range of concentrations. Known and unknown solutions, with the necessary control solutions (2 ml.) were then added to tubes of inoculated medium and incubated at 37° in 5% CO_2-air. An ordinary bacteriological incubator was used; shaking the tubes in a thermostat at 37° did not give a more regular response.

Measurement of streptococcal growth. This was carried out photoelectrically, using a Hilger absorptiometer to obtain the optical densities of the cultures after growth. While the conditions of assay were being chosen (i.e. in producing curves such as those of Fig. 1), portions of about 1·5 ml. of the cultures were taken at intervals, aseptically, with Pasteur pipettes. Cells were used which held *c.* 0·5 ml. of solution but gave an absorbing layer 1 cm. in depth. Densities were normally read after 16–24 hr. incubation, but sometimes also at 40 hr.; in the first case it was not necessary to take specimens aseptically.

Reference curves such as those of Fig. 1*B* were constructed from the readings obtained from the known quantities of glutamine. The glutamine content of each of the tubes containing solutions being assayed was computed by reference to the standard curve. Some results are quoted in Table 2. The standard curves often contained an almost linear portion as illustrated by the full lines in Fig. 1*B*, but sometimes were sigmoid, as shown by the broken line.

It will be observed that the assay could be made sensitive to 1/5 of the quantity of glutamine employed in the present account by using the same method of measurement but 1/5 of the quantity of media.

Specificity of assay. The effect of a number of compounds related to glutamine on growth of the present strain of streptococci has been examined (McIlwain, 1939), without finding any which replaced glutamine. The present medium already contains ammonium glutamate, and the addition of a further 2000 mμmol. of the substance per tube was found to have no effect on the assay of 20 mμmol. of glutamine. Glutamine added to bacterial extracts of the type assayed frequently by McIlwain *et al.* (1948) was satisfactorily recovered, indicating the absence of antagonistic or potentiating substances, but such experiments have not been performed with a wider range of materials.

Table 2 also gives results which show the value of the sulphoxide in assay. Values in the presence of the compound are seen to be much more consistent.

Table 2. *Results of glutamine assay, with and without sulphoxide and added glutamine*

Quantity taken of 1/50 dilution of horse plasma (ml.)	Glutamine found, calculated to nearest 10 mμmol./ml. plasma: By assay without sulphoxide — Reading after 17 hr. growth	By assay without sulphoxide — Reading after 25 hr. growth	By assay with sulphoxide — Reading after 17 hr. growth	By assay with sulphoxide — Reading after 25 hr. growth
2·0	830	710	530	490
1·6	840	830	520	500
1·2	730	770	520	520
0·8	690	750	510	510
Averages:	770	760	520	510

Quantity taken of: Bacterial extract* (ml.)	Glutamine (mμmol.)	Glutamine found, calculated to nearest 0·1 mμmol./ml. extract: Reading after 18 hr. growth — Directly	Reading after 18 hr. growth — After subtracting quantity added	Reading after 26 hr. growth — Directly	Reading after 26 hr. growth — After subtracting quantity added
2·0	0	11·0	—	10·9	—
1·6	0	10·8	—	10·3	—
1·2	0	10·1	—	11·2	—
0·8	0	11·3	—	11·2	—
	Averages:	10·8	—	10·9	—
1·0	25	35·0	10·0	35·0	10·0
0·8	25	35·7	10·7	36·2	11·2
0·6	25	35·9	10·9	34·8	9·8
0·4	25	35·5	10·5	35·0	10·0
	Averages:	35·5	10·5	35·3	10·3

* Prepared by extracting *Staphyloccus aureus* (after treatment similar to that described by McIlwain *et al.* 1948; Table 10) with phosphate buffer pH 7 (4 ml./1·6 g. moist wt. of cells) at 100° for 15 min.; sulphoxide present during assay.

The reason for their absolute values being about 2/3 of those in the absence of the sulphoxide is not known. Acceleration of growth of the assay organism, by compounds unrelated to glutamine, might be expected to have a greater effect on the earlier than on the later readings, but the average values at the two times are found to be approximately equal. It is also possible that there exist in plasma substances which simulate glutamine in the absence of the sulphoxide, or promote its synthesis.

Measurement of response by acid formation. The assay organism converts glucose to lactic acid in a yield of *c.* 95% (McIlwain, 1946). When this was measured by titration, a period of growth of some 3 days gave the most regular response to added glutamine (Fig. 2). As this period is longer than that required for measurement of growth by photometric readings, we have not attempted to base an assay on acid formation, although the type of response is adequate for such an assay.

Weight of streptococci produced by limiting quantities of glutamine. Assay curves such as those of Fig. 1*B* afford data which can be used in calculating the minimum quantity of glutamine required to produce a given quantity of streptococci. Comparison of the optical densities (*a*) and dry weights

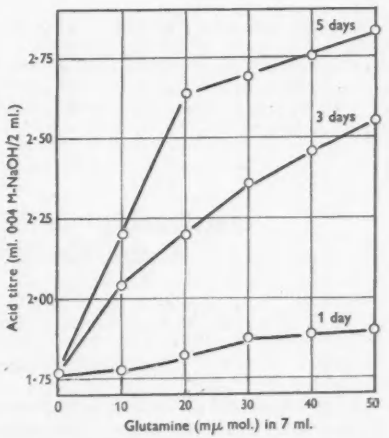

Fig. 2. Acid formed in streptococcal cultures initially containing varying quantities of glutamine, and measured by the alkali required at different times to take 2 ml. portions to a standard pH of about 11. Medium and conditions of growth: as described for photometric assay. Indicator: alizarin yellow G.

(b; mg./ml.) of different batches of the present organisms gave values for b/a of 0·368, 0·345, 0·377, 0·390 and 0·353 (mean: 0·367). The slopes of the initial portions of the lines of Fig. 1*B* show suspensions of optical densities 0·18 (in 16 hr.) to 0·23 (in 24 hr.) to be produced in the first day from 30 mμmol. of glutamine in 7 ml.; 0·015–0·02 mg. of organisms are thus formed per mμmol. of added glutamine. Eight other standard curves have given values differing from this by less than 25%.

SUMMARY

1. A number of substances structurally related to glutamic acid has been prepared, including the hydrazide, $R.CO.NH.NH_2$; its acetone derivative, $R.CO.NH.N{:}C(CH_3)_2$; its benzaldehyde derivative, $R.CO.NH.N{:}CH.C_6H_5$; the hydroxamic acid, $R.CO.NHOH$; and an analogous sulphoxide, $R.SO.CH_3$ and sulphone, $R.SO_2.CH_3$

(R = —$CH_2.CH_2.CH(NH_2).COOH$).

The hydrazide and hydroxamic acid similarly related to aspartic acid have also been prepared.

2. Some of these compounds, and also other substances related to glutamic acid (especially homologues of glutamic acid), had no effect on growth and glycolysis of streptococci. Those formulated above, except the benzaldehyde derivative and the sulphone (which however inhibited *Escherichia coli*), inhibited streptococcal growth. The basis for the action of the hydroxamic acid is unknown, but action of the other compounds on streptococci is related to the metabolism of glutamine or of glutamic acid.

3. Inhibition by the sulphoxide has been applied in the microbiological determination of glutamine. Glutamic acid can to some extent replace glutamine in growth of streptococci in ordinary media, but the sulphoxide (0·01 M) prevents this and results in a prompt and adequately stable growth of the organisms in the presence of 5–40 mμmol. of glutamine in 7 ml. of medium. Photometric measurement of the resulting growth has been used to determine glutamine in natural materials.

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REFERENCES

Archibald, R. M. (1945). *Chem. Rev.* **37**, 161.
Bergmann, M. & Zervas, L. (1933). *Hoppe-Seyl. Z.* **221**, 51.
Borek, E., Miller, H. K., Scheiness, P. & Waelsch, H. (1946). *J. biol. Chem.* **163**, 347.
Fildes, P. (1938). *Brit. J. exp. Path.* **19**, 239.
Hac, L. R., Snell, E. E. & Williams, R. J. (1945). *J. biol. Chem.* **159**, 273.
Hale, J. H. & McIlwain, H. (1947). *Brit. J. exp. Path.* **28**, 346.
Junell, R. (1934). *Ark. Kemi Min. Geol.* **11** B, No. 30.
Knight, B. C. J. G. (1945). *Vit. & Horm.* **3**, 105.
Knight, B. C. J. G. & Fildes, P. (1936). *Biochem. J.* **30**, 1138.
Lewis, J. C. & Olcott, H. S. (1944). *J. biol. Chem.* **157**, 265.
Lyman, C. M., Kuiken, K. A., Blotter, L. & Hale, F. (1945). *J. biol. Chem.* **157**, 395.
McIlwain, H. (1939). *Biochem. J.* **33**, 1942.
McIlwain, H. (1944). *Biochem. J.* **38**, 97.
McIlwain, H. (1946). *Biochem. J.* **40**, 67.
McIlwain, H., Fildes, P., Gladstone, G. P. & Knight, B. C. J. G. (1939). *Biochem. J.* **33**, 223.
McIlwain, H. & Hughes, D. E. (1945). *Biochem. J.* **39**, 133.
McIlwain, H., Roper, J. A. & Hughes, D. E. (1948). *Biochem. J.* **42**, 492.
Madinaveitia, J., Martin, A. R., Rose, F. L. & Swain, G. (1945). *Biochem. J.* **39**, 85.
Toennies, G. & Kolb, J. J. (1939). *J. biol. Chem.* **128**, 399.
Toennies, G. & Kolb, J. J. (1941). *J. biol. Chem.* **140**, 131.
Waelsch, H. & Borek, E. (1947). *Fed. Proc.* **6**, 301.
Waelsch, H., Owades, P., Miller, H. K. & Borek, E. (1946). *J. biol. Chem.* **166**, 273.

Relationships in Streptococci between Growth and Metabolism of Glutamine

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Previous investigation of the behaviour of glutamine as a growth essential for streptococci has suggested its action to have a metabolic basis in two senses. First, requirement for glutamine in growth appeared to depend on the inability of those streptococci which needed added glutamine to synthesize it at an adequate rate. This has now been confirmed by demonstrating the synthesis in organisms not requiring added glutamine, and further by inhibiting such synthesis when growth also has been found to be inhibited. Secondly, glutamine when added to streptococci has metabolic effects, among which has been recognized (McIlwain, 1946 *a*, *b*) a stimulation in glycolysis coupled with the conversion of glutamine to glutamic acid and ammonia. This group of reactions might be that for which glutamine (synthe-

sized by the organisms or added as such) is required by streptococci. To previous evidence suggesting this has now been added two further types of observation: one, that a number of substances related to glutamine, but which are incapable of replacing glutamine in growth, have no effect on glycolysis; and the other, that when the coupled breakdown of glutamine is inhibited, growth of the organisms is also inhibited.

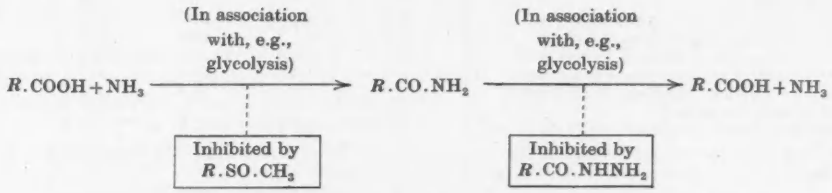

Fig. 1. Points of action of inhibitors in the synthesis and breakdown of glutamine.
R=—$CH_2.CH_2.CH(NH_2).COOH$.

The reactions concerned are summarized in Fig. 1, and evidence is given below for their differential inhibition by two compounds structurally related to glutamic acid, namely, α-amino-γ-methylsulphinylbutyric acid, and γ-glutamylhydrazine. These compounds were selected for detailed study from a series described by Roper & McIlwain (1948). It will be observed from Fig. 1 that the synthesis and breakdown of glutamine are brought about by distinct systems, each associated with energy-yielding reactions such as glycolysis. Other aspects of the association between glutamine and glucose metabolism are reported below.

EXPERIMENTAL

Growth of streptococci, the performance of metabolic experiments with streptococcal suspensions, purification of glutamine, and determination of NH_3 and of glutamine were by methods previously stated (McIlwain, 1946 *a*, *b*) unless others are specified. Casein hydrolysate was that of McIlwain & Hughes (1944). The streptococcal strains employed are described by McIlwain (1946 *a*, *b*) and by Hale & McIlwain (1947).

Disintegration of bacteria followed Wiggert, Silverman, Utter & Werkman's (1940) method, using particles of Pyrex glass about the size of the bacteria. A mass of bacteria packed by centrifuging was cooled in ice and mixed with three times its (moist) weight of the glass, transferred to a dry, ice-cooled mortar and rubbed with maximum hand pressure for 30 sec., scraped together, and the rubbing twice repeated. The resultant paste was extracted in a centrifuge tube with cold M/18 phosphate, pH 7, 1·5 times the moist weight of the original bacteria. Disintegration was observed microscopically and its extent determined by mounting stained films in water instead of oil. The particles of glass could then be seen and the ratio of particles to bacteria was found before and after the grinding.

Extraction by autolysis. Organisms packed by centrifuging were suspended in seven times their moist weight of 0·055 M-phosphate of pH 7, containing 0·36 M-NaF, and left with occasional shaking at 37° for periods of 3–10 days.

Experiments with streptococcal extracts. In addition to the conditions of metabolism referred to above, a medium of the following composition (cf. Utter, Reiner & Wood, 1945) was used in attempting to obtain glycolysis in streptococcal extracts: Na_2HPO_4 and KH_2PO_4 of pH 7·3, 0·01 M; $NaHCO_3$, 0·05 M; $MgCl_2$, 0·008 M; glucose, 0·03 M; hexosediphosphate, 0·0025 M; adenosine triphosphate, 0·0006 M; cozymase (as a specimen of about 40% purity), 0·001 M, with and without nicotinamide, 0·04 M, and in equilibrium with 5% CO_2 in N_2.

Conditions employed in examining the actions of substances on streptococcal growth. A medium based on Bacto-peptone was used (McIlwain, Fildes, Gladstone & Knight, 1939; McIlwain, 1939), inoculated with about 500 cocci/ml. of the different streptococcal strains, and incubated at 37°. The mass of growth in control and experimental tubes was compared visually during the 7 days immediately following inoculation.

Materials. The compounds of Fig. 3 and Table 6 were either obtained commercially, or prepared according to McIlwain (1939). Hynson, Westcott and Dunning Inc., Baltimore, supplied bromsulphalein. Glutamine, glutamic acid and γ-glutamylhydrazine were the natural L-isomers. α-Amino-γ-methylsulphinylbutyric acid was the mixture of isomerides produced from DL-methionine by Roper & McIlwain (1948).

RESULTS

Effects of various circumstances on glutamine breakdown and on streptococcal glycolysis

Before comparing the behaviour of other compounds with that of glutamine, the effects of incidental circumstances on reactions between streptococci and glutamine itself were examined. The actions of glutamine which were studied were (*a*) the rate of glutamine breakdown, especially during anaerobic glycolysis, and (*b*) the associated stimulation of glycolysis.

Preparation of organisms. Results with organisms grown for different periods of time, and in different media, are given in Table 1. Though variations in the rate of NH_3 formation from glutamine occurred,

Table 1. *Variation in response to glutamine with age, medium of growth, and treatment of organisms*

(The Richards strain was employed, and cultures contained 5×10^{-4} M-glutamine unless otherwise indicated.)

Culture medium	Age (hr.)	Dry wt. used/3 ml. (mg.)	Q_{NH_3} (μmol./mg. dry wt./hr.)	Q_{CO_2} without glutamine (μmol./mg. dry wt./hr.)	Q_{CO_2} with glutamine as % of that without	Ratio $\frac{Q_{CO_2}}{Q_{NH_3}}$ in presence of glutamine
1. Casein hydrolysate	12	2·9*	2·55	25·5	126	12·6
2. Casein hydrolysate	26	2·8†	1·07	12·7	128	15·2
3. Broth (no added glutamine)	17	2·7	0·86	11·0	129	16·5
4. Glucose-broth	17	2·7	0·87	11·1	128	16·3
5. Casein hydrolysate; glutamine, 4×10^{-3} M	18	3·2	3·1	20·7	128	8·6
6. Casein hydrolysate; glutamine, 2×10^{-6} M	18	3·8	2·7	18·3	127	8·6
7. Casein hydrolysate; organisms washed once	17	5·0	1·95	15·3	124	9·7
8. Casein hydrolysate; organisms washed thrice	17	5·0	1·90	13·6	119	8·5
9. Casein hydrolysate; organisms washed thrice and incubated in saline‡	17	5·0	1·41	11·8	121	10·1
10. Casein hydrolysate; pyridoxin, 2×10^{-4} M	18	4·3	2·1	15·3	118	8·6

* Containing $2·7 \times 10^8$ viable organisms/mg. dry wt. † Containing $2·3 \times 10^5$ viable organisms/mg. dry wt.
‡ 10 mg. dry wt. of organisms in 10 ml. 0·9% (w/v) NaCl at 37° for 30 min.

these usually roughly paralleled the rates of glycolysis of the different preparations. This emphasized the dependence of glutamine breakdown on glycolysis; it was concluded that the different treatments did not have immediate effects on the reaction with glutamine. The conditions of growth included instances in which glutamine (medium 6, Table 1) or glucose (medium 3) were limiting factors in growth; organisms harvested at 12 hr. (medium 1) were at pH 7, while those harvested at 26 hr. (medium 2) had been at pH *c.* 5 for some hours. The effect of increased pyridoxin on growth was examined (medium 8) as a derivative of it acts as coenzyme in two reactions possibly relevant to glutamine (Gale & Epps, 1944; Gunsalus, Bellamy & Umbreit, 1944; Baddiley & Gale, 1945; Lichstein, Gunsalus & Umbreit, 1945) and the substance sometimes slightly accelerated growth of the 'Richards' streptococci in one of the experimental media, namely, that based on casein hydrolysate.

Concentration of glutamine in reaction-mixture, and time of reaction. The rate of NH_3 formation from glutamine during glycolysis by the 'Richards' streptococci (Table 2A) fell slightly during an hour's reaction, while the rate of glycolysis remained constant. In a separate experiment the streptococci were added to mixtures already containing glutamine and glucose; a relationship similar to that of Table 2A was observed, but with the superposition of short lags before glycolysis and glutamine breakdown reached their steady rates. The ratios of mol. NH_3 formed/mol. glutamine reacting did not markedly change during the experiment, but were between 0·88 and 0·95.

The fall in Q_{NH_3} observed at the end of the experiment of Table 2A was not due to the lowered concentration of glutamine which had then been reached. This is shown in Table 2B, where Q_{NH_3} is seen to be substantially unaffected by a 40-fold variation in glutamine concentration. The range of concentrations of the table cover those of blood plasma and tissues (cf. Archibald, 1945), but do not fall to the values at which glutamine becomes a limiting factor in streptococcal growth.

Table 2. *Kinetics of ammonia production from glutamine*

A. (Glutamine (8·3 μmol.) was added to glycolyzing streptococci (2·5 mg. dry wt.; Richards strain; final volume, 3·5 ml.) at 37° at a time taken as zero, in a series of vessels which were successively placed in ice at the times indicated. Values for Q_{NH_3} are calculated for the successive intervals.)

Time after mixing (min.)	NH_3 (μmol.)	Q_{NH_3} (μmol./mg. dry wt./hr.)
0	0	
		2·6
15	1·67	
		2·4
25	2·66	
		2·1
38·5	3·84	
		2·3
53·5	5·32	
		1·7
70	6·50	

B. (Organisms (Richards streptococci, 2·4 mg. dry wt.) were added at 37° to portions of the normal metabolic mixture containing the quantities of glutamine given below, in a final volume of 3 ml. After 34 min. the mixtures were cooled in ice and NH_3 determined. The values for mean concentrations of glutamine were calculated on the assumption that 1 mol. of glutamine disappeared for each mol. of NH_3 formed. See text, and McIlwain, 1946*b*.)

Initial glutamine (μmol.)	NH_3 formed (μmol.)	Mean glutamine concentration (M $\times 10^4$)
41·2	2·1	134
20·6	2·1	65
10·3	2·0	31·3
5·15	2·1	13·7
2·58	2·1	3·6

(Mean Q_{NH_3} 1·53 μmol./mg. dry wt./hr.)

The effects of varying concentrations of glutamine on the duration and degree of stimulation caused in glycolysis have already been reported (McIlwain, 1946*a*).

Concentration of organisms in reaction mixture. This variable had a major effect on the stimulation of glycolysis. When, in the absence of added glutamine, a suspension of organisms was diluted so that the glycolyzing mixture contained *c.* 0·03 mg. dry weight of bacteria/ml. in place of *c.* 3 mg., their rate of glycolysis expressed as a metabolic quotient (i.e. after allowing for the decreased quantity of organisms) was lowered to about one-half. This occurred with organisms washed once and with others washed three times, and so was not dependent on the dilution of substances only loosely associated with the bacteria. The major effect of glutamine on glycolysis was to make good a large part of the deficiency induced by dilution (Table 3). The stimulation given by glutamine thus decreased with increasing concentrations of streptococci, as is illustrated in Fig. 2. The small stimulations in glycolysis quoted in Table 1 are associated with the relatively large concentration of organisms used in those experiments. As was shown previously (McIlwain, 1946*a*) the stimulation in glycolysis was not dependent on growth; optical density measurements during experiments of Table 3 were not found to change and so confirmed this.

In contrast with the results with streptococci comparable dilution of a suspension of an organism not requiring glutamine in growth did not affect its rate of glycolysis (Fig. 2).

The rates of decomposition of glutamine during glycolysis by 'Richards' streptococci in suspensions of density 1·5 and 0·075 mg. dry wt./ml. were compared, and found to differ by $< 10\%$, though the stimulations of glycolysis in the two cases were markedly different (to 120 and 157 % respectively of controls without added glutamine). Similar results were obtained with 'Buxton' streptococci in suspensions of densities 2·7 and 0·27 mg. dry wt./ml.

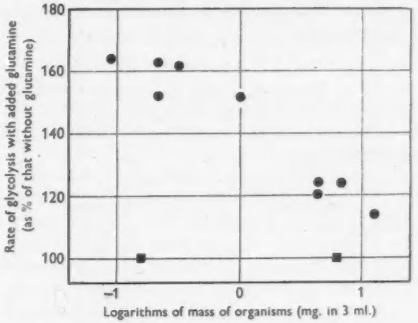

Fig. 2. Relationship between the effect of glutamine on glycolysis, and the concentration of cocci in the glycolyzing mixture. Circles, the Richards streptococcus. Squares, a strain of *Staphylococcus aureus* which did not require glutamine in growth.

Rates of glutamine breakdown and of glycolysis. Streptococcal glycolysis continues for some hours in the absence of added glutamine, though the organisms themselves contain relatively little glutamine (see below). There is therefore no necessarily fixed ratio between rates of glycolysis and of glutamine decomposition. When both substances are

Table 3. *Deficiency induced in glycolysis by dilution of bacterial suspension*

(Richards (R) or Buxton (B) streptococci were added at a time taken as zero to vessels containing the normal metabolic mixture with and without glutamine (20 μmol.) and of final volume 3 ml.)

Organisms; dry wt. (mg./3 ml.)	Change measured during period (min. after commencing reaction)	Q_{CO_2} Without addition (μmol./mg. dry wt./hr.)	(As % of (*a*) in same experiment)	Q_{CO_2} With glutamine (μmol./mg. dry wt./hr.)	(As % of (*b*) in same experiment)	Ratio: $\frac{Q_{CO_2} \text{ with glutamine}}{Q_{CO_2} \text{ without glutamine}} \times 100$
R (batch 1) 13	0–30	10·8 (*a*)	100	11·7 (*b*)	100	109
R (batch 1) 1·08	0–30	7·1	66	10·6	90	150
R (batch 1) 0·09	0–120	4·5	42	7·5	64	167
R (batch 2) 4·4	5–25	18·1 (*a*)	100	22·3 (*b*)	100	123
R (batch 2) 0·22	5–25	14·6	80	21·6	97	148
R (batch 2) 4·4	25–45	17·8 (*a*)	100	21·4 (*b*)	100	120
R (batch 2) 0·22	25–45	11·6	65	19·2	90	166
R (batch 3) 7·7	0–25	18·1 (*a*)	100	21·7 (*b*)	100	120
R (batch 3) 0·38	0–25	11·1	61	18·2	84	164
B (batch 1 washed once) 1·2	0–115	9·35	—	13·0	—	139
B (batch 1 washed thrice) 1·2	0–115	9·20 (*a*)	100	9·60 (*b*)	100	104
B (batch 1 washed thrice) 0·06	0–115	4·7	51	7·0	73	149

present in excess, a relatively stable ratio between the two rates is however observed. The ratio is not markedly dependent on the conditions of growth and treatment of the organisms. Tables 1 and 4 show that values for a given strain vary, but not greatly when it is considered that the values quoted include experiments with independently grown organisms over periods of several months. Beyond such variation, differences characteristic of the strain of organisms can be discerned.

Attempted extraction of NH_3-producing enzymes from streptococci

Though Wiggert *et al.* (1940) obtained by ground glass from *Escherichia coli* extracts which were capable of glycolyzing, such an extract was not obtained from the Richards and group G streptococci. Rubbing which disintegrated 90–99 % of the cells was carried out and extraction of the ground material was performed at pH 5, 7 and 8. Further,

Table 4. *Rates of glutamine breakdown and of glycolysis by haemolytic streptococci*

(Reactions were carried out as described in Table 1; streptococcal strains were β-haemolytic with the exception of the *faecalis* strain.)

Strain; temperature of growth	Temperature of reaction	Q_{CO_2} (μmol./mg. dry wt./hr.)	Q_{NH_3} (μmol./mg. dry wt./hr.)	$\frac{Q_{CO_2}}{Q_{NH_3}}$
R; 37°	30°	9·6	1·28	7·6
R; 37°	37°	17·2	2·26	7·6
R; 37°	42°	20·6	2·55	8·1
R; 25°	37°	16·0	1·92	8·3
B; 37°	37°	6·7–13·1	0·81–2·01	4–14
UC2; 37°	37°	6·8–24	0·23–1·55	13–38
Strep. faecalis; 37°	37°	11·6–9·2	0·45–0·63	18–20·5
TI; 37°	37°	20·6	1·28	16·1

Tables 1 and 4 show no simple stoicheiometric relationship between the reactions in glucose and glutamine. The effect of glutamine in stimulating glycolysis may be expressed in terms of the additional CO_2 evolved while NH_3 is being formed from glutamine. No stoicheiometric relationship however exists between the NH_3 and additional CO_2 which are produced together. This reflects the observations above that (*a*) the percentage stimulation of glycolysis, and hence the additional CO_2, increases with decreasing concentrations of streptococci, while (*b*) the rate of NH_3 formation from glutamine does not markedly vary with concentration of organisms.

the initial, packed, moist organisms were prepared by centrifuging suspensions in the reaction mixture with glucose, glutamine and coenzymes in order to protect such systems as could be stabilized by these substrates.

Both the extracts and residues of ground organisms were examined for their ability to decompose glutamine, but reaction was not observed. It was absent not only in the usual media with and without glucose, but also in more concentrated solutions, buffered with bicarbonate or phosphate, in which the minimum dispersion of enzyme systems took place. In such an experiment, 1 g. moist weight of strepto-

Table 5. *Arginine dihydrolase activity of streptococcal extracts; absence of glutaminase*

(The Richards streptococci were used, ground with glass, and extracted with 0·03 M-phosphate (pH 7). Reactions were carried out as described by McIlwain (1946*a*) in 3 ml. of phosphate saline at 37°.)

Extract; wt. used (mg.)	Dry wt. of bacteria corresponding to portion of extract used (mg.)	Substrate (μmol.)	Q_{NH_3} (μmol./equiv. dry wt./hr.)
A; 142	37	Glutamine, 25	0·02
A; 99	26	Glutamine, 5	0·02 ($Q_{glut.}$ – 0·01)
A; 99	26	Nil	0·02
A; 189	50	Arginine, 20	0·92
B (first extract); 91	6	Arginine, 20	0·85
B (first extract); 91	6	Nil	0·02
B (first extract); 122, heated at 100° for 3 min.	8·2	Arginine, 20	0·02
B (second extract); 282	18	Arginine, 20	0·35

cocci was ground and extracted with 1·5 ml. of a solution containing glucose, bicarbonate, adenosine triphosphate, hexosediphosphate, inorganic phosphate, cozymase and glutamine. An extract (1·1 ml.) was obtained of which 0·25 ml. was mixed with 0·1 ml. further solutions for a metabolic experiment. Glutamine breakdown and glycolysis by the extract were $<5\%$ of that of an equivalent quantity of whole organisms.

The inactivation of streptococcal enzymes by such disintegration was not a general one. The arginine dihydrolase (Hills, 1940) of such extracts was of special interest, as this enzyme leads to formation of NH_3, but independently of glycolysis. Extracts were obtained from the streptococci which were slightly opalescent and contained no cells, and which rapidly decomposed arginine but were without effect on glutamine (Table 5). The first extract, made at pH 7, had, at pH 7, one-third of the activity of the organisms from which it was prepared. About a further one-sixth of the original activity was obtained on a second extraction of the same cells. The remainder was not necessarily lost, as the pH optimum and other properties relating to the activity of the extracted enzyme have not been determined.

Autolysis did not yield from the streptococci an enzyme decomposing glutamine.

Specificity of the glutamine structure in relation to streptococcal glycolysis

Comparison has been made of a number of substances with glutamine with respect to (1) production of NH_3 during glycolysis, and (2) stimulation of glycolysis. Results of the comparison are given in Table 6 except in the cases of a few substances whose effects have been described previously (McIlwain, 1946*a*). Sixteen compounds were examined, using the Richards streptococcus, and a few with a group G organism.

The rate of NH_3 production from glutamine in control experiments was about 2 μmol./mg. dry wt./hr., and with the conditions adopted, production at a rate 1/50 of this could be detected. No material yielded NH_3 at a rate of $>0{\cdot}05$ μmol./mg. dry wt./hr. with the exception of arginine and mixtures containing it; this has been reported previously and, as is noted above, occurred independently of glycolysis (Hills, 1940; McIlwain, 1946*a*).

Stimulation of glycolysis to the extent caused by glutamine was not produced by any compound examined. It was reported (McIlwain, 1946*a*) that NH_3 itself caused a lesser stimulation, and that a few compounds, known or presumed to produce NH_3 under the conditions of the present experiments, also

Table 6. *Effects on streptococcal glycolysis, and on* NH_3 *production, of compounds related to glutamine and to* NH_3

(Organisms (0·5–1 mg. dry wt.) were added to reaction mixtures containing glucose and bicarbonate (final vol., 3 ml.); conditions anaerobic.)

Reaction mixture		Q_{CO_2} as % of the value without addenda, for the same period		Mean Q_{NH_3} (μmol./mg. dry wt./hr.)
Organism	Addenda (μmol.)	0–30 min.	30–60 min.	
	A. Ammonia and simple amines			
R	NH_4Cl, 25	110–118	110–116	—
R	NH_4Cl, 2·5	108–116	107–115	—
R	CH_3NH_2Cl, 25	102	103	—
R	CH_3NH_2Cl, 2·5	103	104	—
R	$(CH_3)_2NH_2Cl$, 25	92	95	—
R	$(CH_3)_2NH_2Cl$, 2·5	103	97	—
R	$C_2H_5NH_2Cl$, 25.	103	97	—
	B. Glutamine and related compounds			
R, G	Glutamine, 2·5–50	140–175	140–180	+1·5–2·5
R, G	L- or DL-Glutamic acid, 2·5–15	97–103	100–106	<0·05
R	Glutaric acid monoamide, 50	101	105	<0·03
R	α-Acetamidoglutaric acid amide, 10, 50	99, 102	96, 105	<0·03
R	DL-Uramidoglutaric acid, 2·5–15	97	98	<0·04
R	Pyrrolidone-α-carboxylic acid, 2·5, 15	96	100	<0·03
R	Pyrrolidone-α-carboxylic acid amide, 2·5, 15	104	102	<0·04
	C. Other amino-acids			
R, G	Asparagine, 25	101	105	<0·03
R	Aspartic acid, 25	96	98	<0·04
R	Proline, 2·5, 20	103	104	<0·03
R	Citrulline, 20	101	114	*c.* 0·05
R	Arginine, 10	104	117	+3·9

stimulated glycolysis to values approximating to those of NH_3. To such compounds may now be added citrulline, though the rate of NH_3 production from this compound was small (Table 6). Dimethylamine and ethylamine were without effect. The action of methylamine may be due to NH_3 in the specimen, which was not especially purified; it can be concluded that any effect of methylamine is less than that of NH_3.

The action of inhibitors on glutamine breakdown, glycolysis and on growth

The effect of further substances on these three processes has been examined to see to what extent the response of the processes to given circumstances are correlated. Correlation would suggest their functional interrelation.

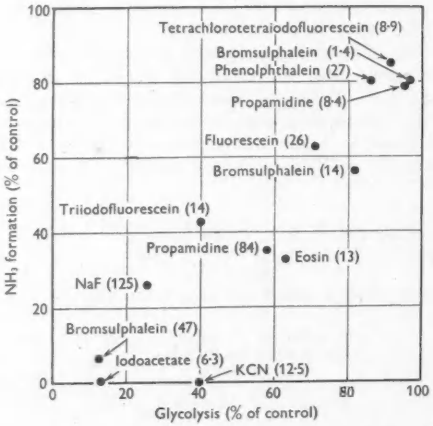

Fig. 3. Comparison of the effects of several inhibitors on streptococcal glycolysis in the presence of glutamine, and on the associated production of NH_3 from the glutamine. The Richards strain was used throughout; numbers beside the names of the inhibitors give their molarities ($\times 10^4$).

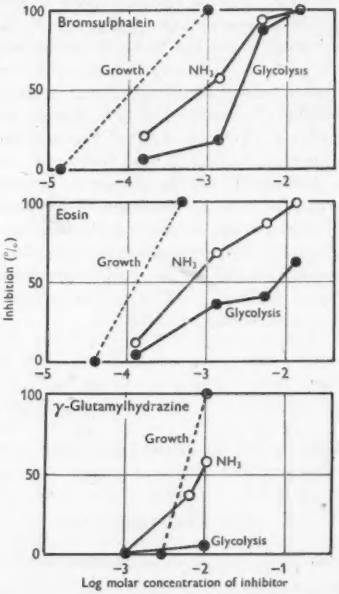

Fig. 4. Inhibition of growth, of the formation of NH_3 from glutamine, and of glycolysis, by different concentrations of bromsulphalein, eosin, and γ-glutamylhydrazine. Growth was studied as described in Table 7, and metabolism as described in Table 1.

Phthaleins. Glutamine hydrolysis by streptococci has not yet been brought about in the absence of glycolysis; in previous attempts to do so by addition of substances inhibiting glycolysis, the glutamine breakdown was also prevented (McIlwain, 1946*a*). As bromsulphalein (a sodium salt of a disulphonic acid of tetrabromophenolphthalein) inhibits a glutaminase of animal tissues (Archibald, 1945), which is not described as being dependent on carbohydrate metabolism, its effect on glutamine metabolism in streptococci has been studied. Bromsulphalein has been found to inhibit the breakdown of glutamine by streptococci, but it inhibited also both glycolysis and growth, often when present in relatively low concentrations. Phenolphthalein, fluorescein, eosin, and a triiodofluorescein were also potent inhibitors of both glycolysis and the breakdown of glutamine in streptococci (Fig. 3).

When the concentrations of the various substances which affect glycolysis and glutamine metabolism were compared, as in Figs. 3 and 4, glutamine breakdown was seen to be the more sensitive of the two processes. It was however the more sensitive to all reagents examined, which showed any selectivity between the two reactions; the reagents studied included monoiodoacetate, cyanide and propamidine. The results of Figs. 3 and 4 thus emphasize the general connexion between glutamine metabolism and glycolysis, without giving any certain evidence that the phthaleins might affect glycolysis through an action on glutamine metabolism.

The phthaleins inhibited also the growth of streptococci, and the effect of varying concentrations of eosin and bromsulphalein on growth was examined. Growth is seen (Fig. 4) to be somewhat more sensitive to these compounds than are glycolysis and glutamine decomposition. The values given in Fig. 4

for the concentrations of the substances affecting growth are approximate only, referring to those of a series of 3-fold dilutions which inhibited completely, or were without visible effect on, growth. They are however adequate to show a marked difference in character of action between the phthaleins and γ-glutamylhydrazine which is discussed below.

Table 7. *Action of varying concentrations of γ-glutamylhydrazine on streptococcal growth*

(Growth was described as prevented if visible opacity did not develop during the 3 days following inoculation under conditions described in the text. Little change was observed during the next 4 days.)

Strain	(*a*) Concentration of glutamine initially present in medium (M)	(*b*) Concentration (M) of glutamylhydrazine		Ratio *b*/*a*	
		Minimal to prevent growth	Maximal allowing growth	Preventing growth	Allowing growth
RNI	2×10^{-3}	10^{-2}	5×10^{-3}	5	2·5
RNI	2×10^{-4}	$2{\cdot}5$–5×10^{-3}	$1{\cdot}25$–$2{\cdot}5 \times 10^{-3}$	12·5–25	6·3–12·5
RNI	2×10^{-5}	$1{\cdot}25$–$2{\cdot}5 \times 10^{-3}$	$6{\cdot}25$–10×10^{-4}	62·5–125	31–50
L	2×10^{-3}	—	2×10^{-2}	—	10
L	2×10^{-4}	2×10^{-2}	10^{-2}	100	50
L	2×10^{-5}	5×10^{-3}	$2{\cdot}5 \times 10^{-3}$	250	125
Strep. faecalis	2×10^{-4}	4×10^{-2}	10^{-2}	200	50
Strep. faecalis	None added	10^{-2}	$2{\cdot}5 \times 10^{-3}$	—	—

The following experiments were carried out in attempting to see whether inhibition of growth by the phthaleins was related to glutamine. Inhibition of the Richards streptococci by bromsulphalein was compared in the presence of minimum glutamine for maximal growth, and in a concentration of 20 times that value. No marked difference was seen. Growth of a group G organism not requiring added glutamine was also inhibited by bromsulphalein, and the level required for inhibition was not affected by the presence of 10^{-3} M-glutamine. Tetrachlorotetraiodofluorescein behaved similarly to bromsulphalein in these two respects.

Effects on growth of compounds structurally related to glutamine. It was anticipated that such compounds might afford examples of inhibition connected with the metabolism of glutamine and of value in studying its effect on growth. Of the many substances examined (Roper & McIlwain, 1948) γ-glutamylhydrazine exhibited a type of action on growth which was promising from this point of view.

Results quoted in Table 7 showed several streptococcal strains to be inhibited by the hydrazide at concentrations of 6×10^{-4} to 2×10^{-2} M. The organisms of Table 7 required added glutamine to varying degrees (see Table 12) but the growth of all could be inhibited by the hydrazide; more of this was however required for inhibition of the less exacting *faecalis* or L strains. Inhibition by the hydrazide was in all cases antagonized by increasing the concentration of glutamine present in the growth medium. The Richards and L strains were examined sufficiently to show that, with increasing concentrations of glutamine in growth media, increasing concentrations of the hydrazide were needed to effect a given degree of inhibition: the hydrazide competed with glutamine. The concentration of hydrazide required for a given effect did not however increase in direct proportion to the increase in concentration of added glutamine. The ratios of Table 7 (and also Fig. 5 which is discussed in detail below) show that a greater preponderance of the hydrazide is needed to inhibit the lower concentrations of glutamine.

The compound had no immediate bactericidal action (Table 8). As its action on growth was associated with glutamine, its effects on the metabolism of glutamine were examined.

Table 8. *Absence of immediate bactericidal action by γ-glutamylhydrazine and α-amino-γ-methylsulphinylbutyric acid*

(The L strain of streptococci grown for 16 hr. was diluted in buffered casein hydrolysate.)

Substance added (M)	Colonies/$1{\cdot}5 \times 10^{-4}$ ml. at times		
	0 hr.	2 hr.	6 hr.
Nil	475	317	244
γ-Glutamylhydrazine (0·02)	—	313	285
α-Amino-γ-methylsulphinylbutyric acid (0·02)	—	348	255

Action of γ-glutamylhydrazine on glycolysis and on the production of NH_3 *from glutamine.* The type of experiment which was performed is illustrated by Table 9. From this, it can be seen that the hydrazide was effective as an agent inhibiting the formation of NH_3 from glutamine by a variety of streptococcal strains. Its action on the glycolysis with which the glutamine breakdown is associated was much less. In several instances, while NH_3 production was

Table 9. *Action of γ-glutamylhydrazine on glycolysis and breakdown of glutamine by streptococci*

(Warburg vessels contained the quantity of hydrazide needed to give the concentrations tested, with bacteria (*c.* 3 mg. dry wt.), salts and $NaHCO_3$ (0·06 M) in 2·0 ml. of water; glutamine (in 0·5 ml. water) was added from a side arm after equilibration with 5 % CO_2 in N_2. A centre well contained yellow phosphorus.)

	Reaction mixture			Products	
Strain	(*a*) Glutamine (M)	(*b*) Hydrazide (M)	Ratio *b*/*a*	Acid formation as % of control without added glutamine and inhibitor	NH_3 formation as % of control with glutamine but without inhibitor
R	4×10^{-3}	2×10^{-2}	5	95	42
R	4×10^{-3}	$6{\cdot}6 \times 10^{-3}$	1·67	102	62
R	4×10^{-3}	2×10^{-3}	0·5	97	96
R	4×10^{-3}	0	0	131	100
L	4×10^{-3}	2×10^{-2}	5	99	14
L	4×10^{-3}	$6{\cdot}6 \times 10^{-3}$	1·67	110	50
L	4×10^{-3}	2×10^{-3}	0·5	113	100
L	4×10^{-3}	0	0	119	100
L	$1{\cdot}6 \times 10^{-2}$	$6{\cdot}6 \times 10^{-3}$	0·42	116	100
Strep. faecalis	$3{\cdot}3 \times 10^{-4}$	$2{\cdot}2 \times 10^{-2}$	6·7	95	33
Strep. faecalis	$3{\cdot}3 \times 10^{-4}$	{Methionine sulphoxide} $3{\cdot}3 \times 10^{-2}$	—	108	105
Strep. faecalis	$3{\cdot}3 \times 10^{-4}$	0	—	108	100

lowered considerably, glycolysis was lowered only to a value about that found in the absence of glutamine.

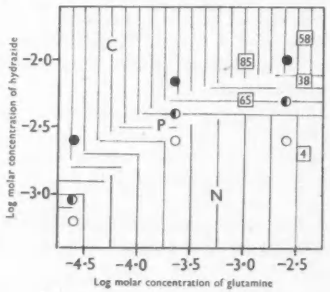

Fig. 5. Comparison of the degrees of inhibition caused in growth and in the formation of NH_3 from glutamine (streptococcus R) by γ-glutamylhydrazine. Circles and shaded areas represent the effects on *growth* of varying concentrations of the hydrazide in the presence of varying concentrations of glutamine. The figures in squares give the percentage inhibition caused in the *formation* of NH_3, at the concentration of glutamine represented by the abscissa value of the square, by the concentration of hydrazide represented by its ordinate value. C and ●, complete; P and ◐, partial; N and ○, no inhibition of growth.

The process of NH_3 formation from glutamine is sensitive to about the same range of hydrazide concentrations, as is growth. Comparison of some of the values of Tables 7 and 9 shows this, and the two processes are compared directly in Fig. 4, which shows that an inhibition of 50 % in the rate of NH_3 formation is associated with a marked inhibition of growth. Fig. 4, in comparing the action of γ-glutamylhydrazine on glycolysis, NH_3 formation and growth, indicates that it has a markedly different type of action from that possessed by the phthaleins and supports the suggestion that the action of the latter compounds on glutamine breakdown is a relatively indirect one.

The substance had little if any action on the assimilation of glutamic acid and glutamine (see below, and Table 11).

Comparison of the action of the hydrazide on growth and on glutamine decomposition. As the major metabolic effect which we have recognized as being brought about by γ-glutamylhydrazine is its action on the formation of NH_3 from glutamine, we have carried out a more extensive comparison between its inhibition of that process and its inhibition of growth. Both inhibitions are affected by the concentration of glutamine present, and are therefore represented in the fashion of Fig. 5. Here it is seen that in mixtures of the hydrazide and glutamine in which the decomposition is inhibited, growth is affected; 40–60 % inhibition of the decomposition is associated with partial inhibition of growth, and 60–85 % with complete inhibition of growth.

Glutamine synthesis by streptococci. Inhibition of the synthesis, and of growth of the organisms

Demonstration of the synthesis. It has been presumed that streptococci not needing glutamine in growth synthesize the compound, though the synthesis has not previously been demonstrated

(McIlwain *et al.* 1939; Fildes & Gladstone, 1939). Attempted determination of glutamine by NH_3 produced on hydrolysis gave negative results with culture fluids, but showed a small quantity of a glutamine-like substance to be associated with streptococcal cells. In order to study changes in the quantity of such material, the microbiological method of determining glutamine which has been described by Roper & McIlwain (1948) was developed. It was then found that a substance, possessing some chemical and microbiological properties of glutamine, could readily be obtained from streptococci grown in the absence of added glutamine. They had therefore synthesized it.

For further study it was desired to bring about the synthesis under conditions more defined than those of a growing culture. Washed streptococcal cells exposed to ammonium glutamate did not yield a glutamine-like substance; but of other materials present during the synthesis by growing cells, glucose, when added with ammonium glutamate, was found to result in the formation of a glutamine-like substance. Experimental conditions for the synthesis are given in Table 10, together with a comparison of the properties of the product with those of glutamine. These properties, the conditions of production of the substance, and its consistent determination as glutamine by a method which itself is highly specific to glutamine make it probable that the material present in the cells is glutamine itself. Of other compounds, those known to be of the lability indicated in Table 10, and perhaps most likely to be present in the cells, are peptides of glutamine such as were studied by Melville (1935); these have however been found not to replace glutamine in growth of the assay organism (McIlwain, 1939). It can be seen from Table 10 that some 10% of glutamine is probably lost during the treatment employed for extracting it from streptococci. No correction for such loss has been applied to the results quoted in this paper, but the conditions of extraction have been rigidly adhered to and applied to washed cells; results have been found to be reproducible to within the limit of accuracy of the assay (about 4% of their value).

Table 10. *Properties of a glutamine-like substance produced by streptococcal cells*

(The material was obtained from 6·5 g. moist wt. of *Streptococcus faecalis*. The cells were shaken aerobically (anaerobiosis made little change in the result) with ammonium L-glutamate (0·16 mmol.), glucose (0·5 mmol.) and Na and K phosphates (1 mmol.) initially at pH 7·6, in 50 ml. water at 37° for 22 min., at which time the pH had become 6·8–7; the cells were washed and the material extracted from them by heating at 100° for 10 min. in 10 ml. 0·005 M-Na and K phosphates at pH 7. It was centrifuged free from cells and contained the equivalent of 160 mμmol. glutamine/ml. Untreated cells extracted in the same way yielded a solution containing 18 mμmol./ml. of glutamine. The suspension of *Clostridium welchii* was prepared and used according to Gale's (1945) description.)

Treatment	Material undergoing treatment	Initial concentration (expressed as glutamine (M))	Material remaining (% of initial quantity)
10 min. at 100°, pH 7	Glutamine	10^{-4}	85–90
	Extract	10^{-4}	90–95
1 hr. at 100°, pH 6, in 0·2 M-phosphate	Glutamine	$1{\cdot}6 \times 10^{-4}$	42
	Extract	8×10^{-5}	32
	Glutamine With extract	8×10^{-5} / 8×10^{-5}	30
20 min. at 100° in N-H_2SO_4	Glutamine	8×10^{-5}	<5
	Extract	8×10^{-5}	<5
30 min. at 37° and pH 4·5 with suspension of *Cl. welchii*	Glutamine	10^{-4}	<4
	Extract	8×10^{-5}	<4

Kinetics. The assimilation of glutamic acid and glutamine. Gale (1947) has shown that an energy-yielding reaction such as glycolysis is required for entry of glutamic acid or glutamine into streptococcal cells, and during the present experiments ample confirmation of these observations has been found. The rate and extent of such assimilation is clearly relevant to glutamine synthesis and we have, therefore, followed assimilation while studying glutamine synthesis. Total glutamic acid and glutamine have been determined by the decarboxylase preparation from *Clostridium welchii* (Gale, 1945). Though this preparation liberates CO_2 from glutamic acid and from glutamine at different rates, we did not find the difference adequate to form the basis of a method for determining glutamine in the presence of glutamic acid in streptococcal extracts. The reagent was therefore used in quantity sufficient to determine both substances, and glutamine determined separately by the method described above. The quantity of glutamine found in cells was usually a fraction only of the quantity of glutamic acid. Both determinations were made after the extraction described in Table 10.

Several experiments on the course of entry of glutamic acid and glutamine are illustrated in Fig. 6. In these, glutamic acid was usually supplied together with 1 equiv. of NH_3, at concentrations between 8×10^{-3} and 5×10^{-4} M. Initial concentrations of glutamine were similar. Glucose was usually present in excess of the quantity metabolized in the 20–45 min. experimental period, and phosphate buffer in quantity adequate to maintain the pH between 7·6 and 7·0. The quantity of streptococci used was between 3 and 10 mg. dry wt./ml. of reaction mixture, but in Fig. 6 results are expressed as the quantity of glutamic acid or glutamine entering unit weight of cells at various times; the

slopes of the lines joining experimental points therefore correspond to metabolic quotients.

Entry of glutamic acid and glutamine into the *faecalis* strains are compared in Fig. 6. The initial rates of entry which were observed in the two compounds were similar and corresponded to between 0·2 and 0·5 μmol./mg. dry wt./hr. The experimental arrangements were such that these were not necessarily the maximal rates of which the organisms were capable, as the first reading was commonly taken after 8 min. exposure, and between it and the second reading a marked fall in rate of assimilation frequently occurred. A smooth curve through the experimental points of Fig. 6 suggested maximal initial rates approaching 1 μmol./mg. dry wt./hr. in the presence of initial concentrations of glutamic acid or glutamine of $5{\cdot}5–8 \times 10^{-3}$M. During assimilation, glycolysis was proceeding at the rate of about 10–15 μmol./mg. dry wt./hr. (Table 11). Rate of entry varied with the concentration of added glutamic acid, in the manner indicated in Table 11. The initial rate of assimilation of glutamic acid from a solution $5{\cdot}5 \times 10^{-3}$M was not found to be affected by increase of the concentration of ammonium ion from 5·5 to $27{\cdot}5 \times 10^{-3}$M. The final concentration of glutamine plus glutamic acid which was reached in cells was frequently greater when glutamine was supplied than when ammonium glutamate was used. This is seen in two instances in Fig. 6, and was most often found at the end of experiments in which the supply of glucose was failing.

Kinetics. The breakdown of glutamine during assimilation experiments. Streptococci rapidly break down glutamine when this is added to them in the presence of glucose. Before studying the possible

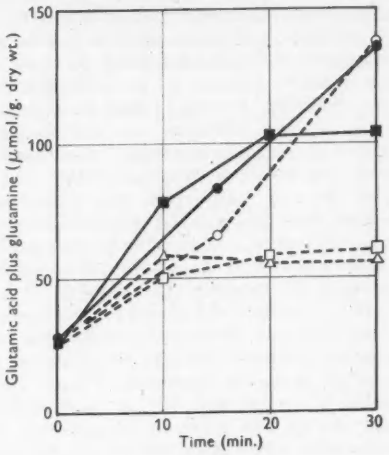

Fig. 6. The course of entry of glutamic acid and glutamine into three batches of cells of *Streptococcus faecalis*. Suspensions of bacteria were divided into portions containing *c.* 0·5 g. wet weight of cells, and these shaken at 37° with 15–20 ml. of 0·08M-Na and K phosphates of initial pH 7·5 and containing 0·017M-glucose and 0·008M-ammonium glutamate or glutamine. Cells were separated after the times indicated, washed, and their content of glutamic acid plus glutamine compared with that of a sample of untreated cells, after the extraction of Table 10. Full lines and black points, uptake following addition of glutamine. Broken lines and white points, uptake following addition of glutamic acid. Points of the same shape refer to experiments carried out simultaneously and with the same batch of cells.

Table 11. *Assimilation of glutamic acid from different initial concentrations; the course of assimilation and of lactic acid formation*

(The organisms and reaction mixtures (18 ml.) were as described in Fig. 6. Organisms *A* contained initially 1·7 μmol., organisms *B*, 2·1 μmol. and organisms *C*, 1·35 μmol. of glutamic acid in the quantity of cells employed below; each contained <0·02 μmol. of glutamine.)

Batch and dry wt. of streptococci (mg.)	Glutamic acid added		Glutamic acid assimilated (μmol.) in *t* min.		Glutamic acid remaining in solution (by difference)		Lactic acid formed (μmol.)
	(μmol.)	(M × 10^{-4})	(*t*)	(μmol.)	(μmol.)	(M × 10^{-4})	
A, 120	54	30	8	3·75	50·2	28·0	294
A, 120	18	10	8	2·40	15·6	8·7	315
A, 120	9	5	8	2·00	7·0	3·9	—
A, 120	0	0	8	—	—	—	289
B, 168	100	62·5	8	1·35	99	55·0	223
B, 168	100	62·5	15	1·95	98	54·5	468
B, 168	100	62·5	30	2·30	98	54·5	502
C, 188	50	—	10	1·35	48·6	27·0	342
C, 188	50; with glutamylhydrazine, 500	30	10	1·45	48·5	26·9	322
C, 188	0	—	10	0	0	0	349
C, 188	0; with glutamine, 50	0	10	2·95	—	—	410
C, 188	0; with glutamine, 50, and glutamylhydrazine, 500	0	10	2·75	—	—	352

synthesis of glutamine, experiments were carried out to see whether any accumulation of glutamine within the cells could be demonstrated when the compound itself was added. Transitory accumulation has been found. Fig. 7 shows this. Here the concentration of glutamic acid plus glutamine rises during the first 20–40 min., and does not fall during the experimental period. Glutamine itself, however, falls in concentration after 10 min. Short experiments are therefore likely to be necessary to demonstrate its synthesis.

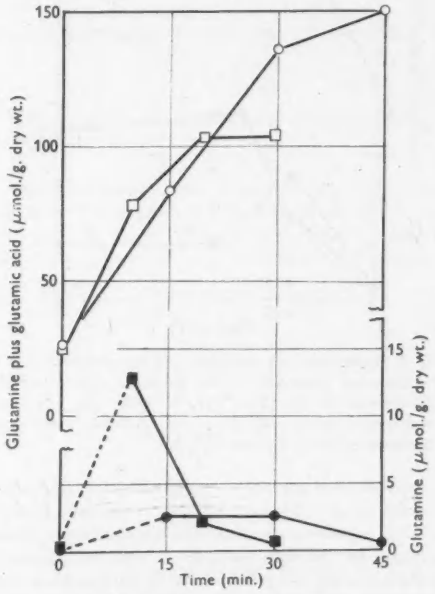

Fig. 7. The temporary accumulation of glutamine, and appearance of glutamic acid, in *faecalis* streptococci exposed to glutamine and glucose. Experimental arrangement, as described in legend to Fig. 6. Black points, glutamine. White points, glutamic acid plus glutamine. Points of the same shape refer to experiments carried out simultaneously and with the same batch of cells.

Kinetics. The synthesis of glutamine from ammonium glutamate. Fig. 8 shows incubation of *faecalis* streptococci with glucose and ammonium glutamate to result in a rise and then a fall of intracellular glutamine. The maximum quantity observed was only 1 mol. % of the ammonium glutamate added and 3 mol. % of the glutamic acid present in the cell at the same time. These values are to be compared with those of Fig. 6 describing the behaviour of added glutamine. Values for the same batch of organisms are available, and here the maximum accumulation observed was 8·5 % of the glutamine added, and 16 mol. % of the intracellular glutamic acid. Synthesis is thus slower than is the entry of glutamine, but it is not known what reduction in the rate of entry would be necessary to give the observed accumulation. The maximum rate of accumulation which we have observed in synthesized glutamine is 0·02 μmol./mg./hr., in cells capable of decomposing added glutamine at the rate of 0·6 μmol./mg./hr.

Connexion between the accumulation of synthesized glutamine in several streptococcal strains and their need for added glutamine in growth. Synthesis of glutamine has been observed in many streptococcal

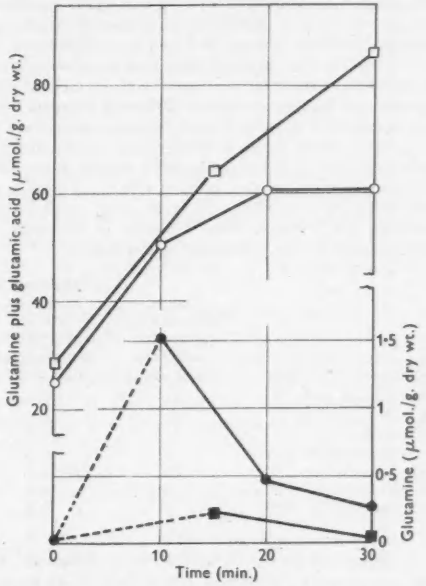

Fig. 8. Accumulation of synthesized glutamine by *faecalis* streptococci. Experimental conditions, as legend to Fig. 6, employing ammonium glutamate with no added glutamine. Symbols, as legend to Fig. 7.

strains in addition to those of Fig. 8. In most cases accumulation was not followed kinetically, but the maximum values which have been observed during short experimental periods are quoted in Table 12. They are seen to vary inversely with the organisms' need for glutamine in growth. This need has been expressed as the increased lag observed in their growth, following the withdrawal of glutamine and the substitution of ammonium glutamate; details are given elsewhere (Hale & McIlwain, 1947). When so expressed organisms most exacting with respect to glutamine gave a value for increased lag (a totalled value under a set of arbitrarily chosen conditions) of about 35 days, and organisms not requiring added glutamine gave a value of zero.

Table 12 shows that the exacting organisms accumulated glutamine at a very slow rate, if at all; while non-exacting strains showed marked synthesis.

Table 12. *Variation in the accumulation of synthesized glutamine by several streptococcal strains*

(Organisms were grown in 1 or 0·5 l. of the medium based on casein hydrolysate and yeast preparation, and, in order to ensure uniformity of growth conditions, glutamine (10^{-5} M) was present in all cases although several organisms did not require its addition for their growth. At 14–24 (usually 16) hr., cells were harvested, washed twice in saline and portions representing about 0·5 g. wet wt. of organisms were used in 16 ml. of solution which contained ammonium glutamate, 0·003 M; glucose, 0·0125 M and phosphates at pH 7·6, 0·085 M. The mixtures were shaken aerobically at 37° and at 10 and 20 min. were cooled with ice and the cells separated and washed with saline. Their cell contents were freed as described in Table 10 and glutamine determined in the extracts. From values so obtained were subtracted the small quantities of glutamine initially present in the cells and determined on another portion which had been kept cold during the experiment. In instances in which it was examined, the solution after exposure of the cells to ammonium glutamate, contained no glutamine.)

Streptococcal strain	Lag on withdrawing glutamine (arbitrary total, days)	Maximum observed rate of accumulation of glutamine (μmol./g./hr.)
6176; β-haemolytic	31	<0·1
RNI; β-haemolytic	26	<0·2
6177; β-haemolytic	8	<0·1
L; β-haemolytic	3	0·3
6181; β-haemolytic	0	2·6
Faecalis; laboratory strain	0	5·3
Faecalis; N.C.T.C. 6796	0	2·2

Inhibition of growth by methionine sulphoxide. Of the compounds examined by Roper & McIlwain (1948), one was initially found to inhibit the growth of a *faecalis* strain of streptococci but not that of the Richards strain. This was α-amino-γ-methylsulphinylbutyric acid, the sulphoxide derived from methionine. The basis for its differential action was found to lie not in the organisms examined but in their conditions of growth; the *faecalis* strain, being non-exacting towards glutamine, was grown without added glutamine while the Richards strain was necessarily grown with glutamine. When the *faecalis* strain was grown in the presence of glutamine, α-amino-γ-methylsulphinylbutyric acid failed to inhibit it. Similar results have been reported with respect to lactobacilli by Waelsch, Owades, Miller & Borek (1946) and Borek, Miller, Scheiness & Waelsch (1946). We found the action of the sulphoxide on streptococci to be unaffected by cystine, methionine and homocystine. The relation of its action to glutamine thus showed a certain specificity. The compound was not markedly bactericidal (Table 8); quantitative details of its action on growth are given below.

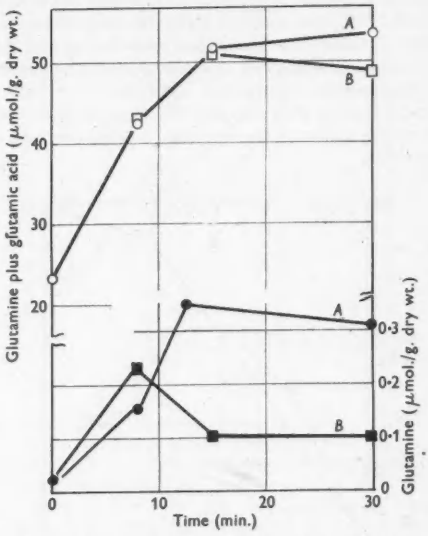

Fig. 9. Assimilation of glutamic acid and accumulation of synthesized glutamine in the presence of excess NH_3. Conditions as legend to Fig. 6, with: (*A*), 100 μmol. ammonium glutamate; and (*B*), 100 μmol. ammonium glutamate plus 500 μmol. NH_4Cl.

Inhibition of glutamine synthesis by the sulphoxide. Waelsch *et al.* (1946) suggested, on the basis of their growth experiments, that the sulphoxide affected lactobacilli through inhibiting their synthesis of glutamine. We have been able to demonstrate that the sulphoxide lowers the quantity of glutamine, formed from ammonium glutamate and glucose, and accumulating in streptococci under the experimental circumstances described in Table 13.

Many processes are occurring during such experiments, but the action of the sulphoxide was found to be a relatively specific one: the assimilation of glutamic acid is seen to be reduced to a smaller extent; in different experiments (Table 9) glycolysis was shown to be unaffected, and the breakdown of glutamine to glutamic acid not to be accelerated by the sulphoxide. It is likely, therefore, that the effect of the sulphoxide on the accumulation of glutamine is due to an inhibition of its synthesis.

Comparison of the effect of the sulphoxide on glutamine synthesis and on growth. The concentrations of methionine sulphoxide required for inhibition of growth were found to be comparable to those causing inhibition of the synthesis of glutamine. Both inhibitions depended also on the concentration of

Table 13. *Action of methionine sulphoxide on the accumulation of synthesized glutamine*

Organism	Reaction mixture*: Ammonium glutamate (M)	Reaction mixture*: Methionine sulphoxide (M)	Glutamic acid + glutamine of cells (μmol./g. dry wt.)	Glutamine of cells (μmol./g. dry wt.)	Glutamine accumulation in presence of sulphoxide as % of value in its absence $\left(\frac{c-a}{b-a}\right)\times 100$
Streptococcus faecalis, laboratory strain	0	0	52	0·30 (*a*)	—
	$2{\cdot}8\times10^{-3}$	0	82	1·00 (*b*)	—
	$2{\cdot}8\times10^{-3}$	0·028	76	0·65 (*c*)	50
	$0{\cdot}55\times10^{-3}$	0	79	0·65 (*b*)	—
	$0{\cdot}55\times10^{-3}$	0·028	73	0·40 (*c*)	28
Streptococcus faecalis, N.C.T.C. 6459	0	0	15·9	0·29 (*a*)	—
	3×10^{-3}	0	79	13·2 (*b*)	—
	3×10^{-3}	0·1	43	0·29 (*c*)	0
	3×10^{-3}	0·033	—	0·57 (*c*)	2

* The reaction mixture in all cases contained approximately the quantity of organisms, glucose, and phosphate which are quoted in Fig. 6.

glutamic acid which was present at the same time, and their comparison is therefore presented in the form given in Fig. 10. This shows that decrease in the rate of accumulation of synthesized glutamine to

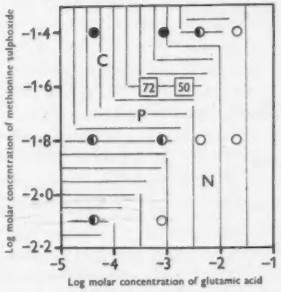

Fig. 10. Comparison of the degrees of inhibition caused in growth and in the accumulation of synthesized glutamine, by α-amino-γ-methylsulphinylbutyric acid. Manner of representation: see Fig. 5.

about half its normal value results in a partial inhibition of growth; this becomes nearly complete when the accumulation is reduced to about a quarter of its normal value. It is therefore feasible that the inhibition of growth is caused by inhibition of glutamine synthesis.

DISCUSSION

The course of glutamine metabolism

It has been shown that synthesis and breakdown of glutamine occur in streptococci in accordance with the reactions of Fig. 1. These reactions are indispensable to most streptococcal strains. Both in growing and in non-proliferating organisms they are associated with other processes such as glycolysis, and one reason for this lies in the requirement for a source of energy for the assimilation of glutamic acid and glutamine (Gale, 1947). The following considerations show, however, that this is not the only type of association between glutamine metabolism and glycolysis.

Some naturally-occurring streptococci do not synthesize glutamine, and in others the synthesis can be inhibited by α-amino-γ-methylsulphinylbutyric acid. In these cases added glutamine allows growth, by providing the product of the system which is missing or inhibited. This confirms our previous knowledge of the importance of the first reaction of Fig. 1. But need for the second reaction is not obviated by providing glutamic acid and NH_3. It is probable, therefore, that the second reaction is more complicated than indicated in Fig. 1 and other data support this view. First, glutamine stimulates streptococcal glycolysis. Secondly, the streptococcal system leading to production of NH_3 from glutamine may be contrasted with one of *Proteus morganii* (McIlwain, 1946c). This is easily extracted from the cells and does not require glucose for its activity. Glycolysis by *Pr. morganii* is unaffected by added glutamine.

Quantitative comparisons of processes involving glutamic acid in proliferating and non-proliferating bacteria

Four such comparisons, linking biological and chemical processes in streptococci, are made below on the basis of data from this and from other papers. The general problem which we wish to answer is whether the changes observed in non-proliferating cells are adequate in type and magnitude to form a basis for the behaviour of the organisms during growth.

Assimilation. The first problem is presented by the finding (Table 14) that streptococcal cells contain about 0·28–0·34 μmol./mg. of glutamic acid combined in the cell structure, together with a more variable quantity of some 0·01–0·1 μmol./mg. as free glutamic acid within the cell. The minimum rate of assimilation necessary to cause this accumulation can be calculated as follows. If q = μmol. of substance found per mg. of cells in a culture growing exponentially, and Q = the instantaneous rate of assimilation of substance in μmol./generation time per mg., then by derivation such as that of Buchanan & Fulmer (1930), it can be shown that $q = Q \log_e 2$. The rates of assimilation necessary to account for the above cell contents of glutamic acid in cultures of a generation time of 20 min. are therefore

$$0{\cdot}6\ (= 0{\cdot}29 \times 0{\cdot}70 \times 3) \text{ to } 0{\cdot}9\ \mu\text{mol./mg./hr.}$$

Observed values for the rates of entry of glutamic acid and glutamine into non-proliferating streptococci have been collected in Table 14. They vary with the quantity of free glutamic acid and glutamine already present in the cells (see below), and the rates in cells of low free glutamic acid agree reasonably well with those calculated above as necessary for growth with generation time of 20 min. The process, in the form in which it has been observed in non-proliferating suspensions, is therefore adequate to provide the quantity of glutamic acid found in cells. There is not, however, a large margin between the quantity observed to enter, and that which is required to enter for the production of cells of the observed composition at the rate of one generation in each 20 min. Also, the cells do not appear capable of assimilating and storing quantities of glutamic acid or glutamine which are large in comparison with that combined in their substance; the rate of assimilation falls as cell content increases. Figs. 7, 8 and 9 show this (see also Gale, 1947). The quantity found as free acid has rarely been one-third that existing combined, presumably as protein. These observations lead to the expectation that inhibition of reactions concerned with streptococcal glutamic acid will be reflected relatively promptly in the generation time of

Table 14. *Rates of reactions which involve glutamic acid and glutamine, in growing and in non-growing streptococci*

Process	Organism	Rate at 37° (μmol./mg. dry wt./hr.)	Source of data; notes
Directly observed in non-proliferating suspensions, with glucose			
Assimilation of glutamic acid	*Faecalis*	0·40	Gale (1947); external glutamic acid, 9×10^{-3} M; initial streptococcal glutamic acid, 6 μmol./g.
Assimilation of glutamic acid	*Faecalis*	0·25	Fig. 6; external glutamic acid, 8×10^{-3} M; initial streptococcal glutamic acid, 25 μmol./g.
Assimilation of glutamine	*Faecalis*	0·72	Gale (1947); external glutamine, 9×10^{-3} M; initial streptococcal glutamic acid, 6 μmol./g.
Assimilation of glutamine	*Faecalis*	0·40	Figs. 6 and 7; external glutamine, 8×10^{-3} M; initial streptococcal glutamic acid, 25 μmol./g.
Conversion of glutamine to glutamic acid and NH_3	R	1·3–2·5	Table 4
Conversion of glutamine to glutamic acid and NH_3	*Faecalis*	0·5–0·6	Table 4
Accumulation of glutamine formed from ammonium glutamate	*Faecalis*	0·01	Fig. 8; organisms decomposed added glutamine at 0·6 μmol./mg./hr.
Rates calculated from content of cell or cell yield, as necessary to permit doubling of size of culture each 20 min.			
Assimilation of the glutamic acid which is found free in cells	*Faecalis*	0·02	Table 11; Gale (1947). Glutamic acid-poor cells of content 6–10 μmol./g.
Assimilation of the glutamic acid which is found free in cells	*Faecalis*	0·15	Table 13; Gale (1947). Glutamic acid-rich cells of content 50–100 μmol./g.
Assimilation of glutamyl residues of cell structure	*Faecalis*	0·60	Gale (1947); from cell content of 0·28 μmol./mg.
Assimilation of glutamyl residues of cell structure	—	0·66	Pollack & Lindner's (1942) estimate of cell content of glutamic acid (5%)
Assimilation of minimum quantity of glutamine permitting growth of exacting streptococci	R	0·12	Roper & McIlwain (1948)

growing cultures, and instances of this are discussed below.

Glutamine synthesis. In the growth of non-exacting streptococci, the importance of the synthesis of glutamine has previously been indicated by indirect evidence, but the process itself has not been demonstrated. The accumulation of glutamine in cells provided with ammonium glutamate and glucose has now been found to be transitory only. If the maximum observed rate is expressed as a metabolic coefficient, it corresponds to a value of +0·01 μmol./mg./hr. As under such conditions glutamine is rapidly reconverted to ammonium glutamate, the rate of accumulation is unlikely to represent a rate of synthesis. Nevertheless, Table 12 shows that the rates of accumulation of glutamine in a series of streptococcal strains are inversely related to their need for added glutamine in growth. We may attempt to estimate the minimal needs of a streptococcus for glutamine (in distinction from glutamic acid) as follows. In an earlier paper (Roper & McIlwain, 1948) on the assay of glutamine, experimental conditions are chosen in which exacting streptococci are provided with excess ammonium glutamate but limited quantities only of glutamine, and are prevented from synthesizing glutamine from the ammonium salt. The quantity of organisms formed per unit weight of glutamine was found to be 15–20 mg. dry wt./μmol. of glutamine. 1 μmol. of glutamine plus glutamic acid would be contained in 2–3 mg. organisms of the glutamine plus glutamic acid content of those of Table 14. We may conclude that the exacting organisms require at least one-fifth of their glutamyl residues as glutamine, and if non-exacting organisms make similar use of glutamine, synthesis at the rate of about 0·12 μmol./mg./hr. would be required. This is much higher than the observed rate of accumulation.

Inhibition of glutamine accumulation by α-amino-γ-methylsulphinylbutyric acid. Although the observed rate of accumulation of glutamine is thus a fraction only of the probable rate of its synthesis, the sulphoxide affects the accumulation in a range of concentration closely paralleling that of its action on growth. Fig. 10 presents this well. We have also on occasions observed the sulphoxide to inhibit the assimilation of glutamic acid (Table 13), but at present express no opinion on possible causal relations between these effects.

Inhibition of glutamine breakdown. It appears from the similar comparison of Fig. 5 that inhibition of the conversion of glutamine to glutamic acid and NH_3 by the γ-hydrazide of glutamic acid also leads to a parallel inhibition of growth. This confirms the view, derived above, that this reaction in normal growth is associated with other changes, for glutamic acid and NH_3 do not prevent the action of the hydrazide on growth. Nevertheless, when the change, which in resting cells produces these substances, is inhibited, growth is also inhibited. Both the synthesis and breakdown of glutamine are thus of functional importance to streptococci.

SUMMARY

1. (*a*) The rate of glycolysis (per unit weight of organisms) by non-proliferating suspensions of β-haemolytic streptococci, which required glutamine for growth, fell on reducing the quantity of organisms in suspension below values of about 3 mg. dry wt./ml.

(*b*) Glutamine largely made good this deficiency, but sixteen substances related to glutamine in various ways were ineffective in doing this.

(*c*) During its action in glycolysis, ammonia was formed from glutamine, but related compounds added in place of glutamine (e.g. asparagine, glutaric acid amide or pyrrolidone-α-carboxylic acid amide) did not lead to ammonia formation.

(*d*) The system responsible for glutamine breakdown was not obtained in cell-free form, though arginine dihydrolase was so obtained.

2. (*a*) γ-Glutamylhydrazine inhibited the production of ammonia from glutamine during streptococcal glycolysis. The concentration of the hydrazide required for this effect increased with increase in the concentration of glutamine present; when this concentration was between 0·4 and $1·6 \times 10^{-2}$ M, the hydrazide at $0·2–2 \times 10^{-2}$ M gave about 50% inhibition in the rate of ammonia formation.

(*b*) A similar range of concentrations of the reagents gave partial inhibition of growth, and again greater concentrations of the hydrazide were required in the presence of greater concentrations of glutamine.

(*c*) Many substances (including a number of phthaleins), which inhibited glycolysis, inhibited also the formation of ammonia from glutamine, and inhibited growth, but the relative sensitivities of these processes were different from those found in the case of the hydrazide, and supported the suggestion that the primary action of the hydrazide was in the breakdown of glutamine, and that this led to inhibition of growth.

3. (*a*) In the presence of glucose, glutamic acid or glutamine entered the cells of non-proliferating streptococci at rates of about 0·5–1 μmol./mg. dry wt./hr., which is adequate to account for the production of successive generations of cells of the observed glutamic acid content of 0·3–0·5 μmol./mg. and growing with a generation time of 20 min.

(*b*) Streptococcal cells contained a small quantity of material with the chemical and microbiological properties of glutamine. The quantity in streptococci which did not require added glutamine in growth could be increased by brief incubation with ammonium glutamate in the presence of glucose.

(*c*) Glucose also led to breakdown of glutamine, and the accumulation in streptococcal cells was found to be transitory only. This applied to glutamine added as such, as well as to that produced from ammonium glutamate and glucose. The maximum observed rate of accumulation of synthesized glutamine was about 0·01 μmol./mg./hr. in cells capable of decomposing added glutamine at the rate of 0·6 μmol./mg./hr. The rate was much lower, and probably zero, in organisms requiring added glutamine in growth.

(*d*) α-Amino-γ-methylsulphinylbutyric acid inhibited the accumulation of synthesized glutamine in non-exacting streptococci. In one strain, $2{\cdot}8 \times 10^{-2}$M-solutions of the sulphoxide lowered the initial rate of accumulation from $2{\cdot}8 \times 10^{-3}$M-ammonium glutamate by 50%. These concentrations of reagents led to partial inhibition of growth of the organism, and the action of other concentrations on growth and metabolism were similarly correlated. The compound was without action on growth in the presence of glutamine and its action on growth in the presence of glutamate was presumably due to inhibition of glutamine synthesis. Both the synthesis and breakdown of glutamine are thus of functional value to the organisms.

We are greatly indebted to Miss E. Ellis for assistance during these investigations.

REFERENCES

Archibald, R. M. (1945). *Chem. Rev.* **37**, 162.
Baddiley, J. & Gale, E. F. (1945). *Nature, Lond.*, **155**, 727.
Borek, E., Miller, H. K., Scheiness, P. & Waelsch, H. (1946). *J. biol. Chem.* **163**, 347.
Buchanan, R. E. & Fulmer, E. I. (1930). *Physiology and Biochemistry of Bacteria*, **3**, 156. London: Baillière, Tindall and Cox.
Fildes, P. & Gladstone, G. P. (1939). *Brit. J. exp. Path.* **20**, 334.
Gale, E. F. (1945). *Biochem. J.* **39**, 46.
Gale, E. F. (1947). *J. gen. Microbiol.* **1**, 53.
Gale, E. F. & Epps, H. M. R. (1944). *Biochem. J.* **38**, 232.
Gunsalus, I. C., Bellamy, W. D. & Umbreit, W. W. (1944). *J. biol. Chem.* **155**, 685.
Hale, J. H. & McIlwain, H. (1947). *Brit. J. exp. Path.* **28**, 346.
Hills, G. M. (1940). *Biochem. J.* **34**, 1057.
Lichstein, H. C., Gunsalus, I. C. & Umbreit, W. W. (1945). *J. biol. Chem.* **161**, 311.
McIlwain, H. (1939). *Biochem. J.* **33**, 1942.
McIlwain, H. (1946*a*). *Biochem. J.* **40**, 67.
McIlwain, H. (1946*b*). *Biochem. J.* **40**, 460.
McIlwain, H. (1946*c*). *J. Path. Bact.* **58**, 322.
McIlwain, H., Fildes, P., Gladstone, G. P. & Knight, B. C. J. G. (1939). *Biochem. J.* **33**, 223.
McIlwain, H. & Hughes, D. E. (1944). *Biochem. J.* **38**, 187.
Melville, J. (1935). *Biochem. J.* **29**, 179.
Pollack, M. A. & Lindner, M. (1942). *J. biol. Chem.* **143**, 655.
Roper, J. A. & McIlwain, H. (1948). *Biochem. J.* **42**, 485.
Utter, M. F., Reiner, J. M. & Wood, H. G. (1945). *J. exp. Med.* **82**, 218.
Waelsch, H., Owades, P., Miller, H. K. & Borek, E. (1946). *J. biol. Chem.* **166**, 273.
Wiggert, W., Silverman, M., Utter, M. F. & Werkman, C. H. (1940). *Iowa St. Coll. J. Sci.* **14**, 179.

The Estimation of the Leucine Isomers in Protein Hydrolysates by Infrared Analysis

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Certain of the monoamino-acid constituents of protein hydrolysates may be estimated by partition chromatography (Martin & Synge, 1941; Gordon, Martin & Synge, 1943; Tristram, 1946). This method, however, does not effect any separation of leucine $(CH_3)_2CH.CH_2.CH(NH_2)COOH$ and isoleucine $CH_3.CH_2.CH(CH_3).CH(NH_2)COOH$, the acetyl derivatives of which have identical properties under the conditions of the Martin & Synge technique. The present investigations were directed towards obtaining a rapid and accurate spectroscopic method for analyzing the isoleucine/leucine ratio in the leucine fractions separated from protein hydrolysates by partition chromatography.

The possibility of analyzing a mixture of leucine and isoleucine by means of differences in their vibration spectra was first suggested by Edsall (1938) in work on the Raman spectra of amino-acids, but, as Edsall noted, the Raman technique presents many difficulties in such a problem. In the course of spectroscopic work on amino-acids, peptides and proteins, we recorded the infrared spectra of leucine and isoleucine and their acetyl derivatives, the latter for the first time. As might be anticipated from the

differences in the hydrocarbon skeletons of such isomers, marked differences were found between the spectra of leucine and isoleucine, and between those of their acetyl derivatives. The spectra of the latter are illustrated in Fig. 1, from which it is clear that the 714 cm.$^{-1}$ band of acetyl-L-leucine and the 736 cm.$^{-1}$ band of acetyl-L-isoleucine should provide a ready means of distinguishing these two isomers and estimating their relative abundance in a mixture. Using partition chromatography (Gordon *et al.* 1943; Tristram, 1946) it is possible to isolate a representative mixture of the leucine isomers as their acetyl derivatives. For this reason, the infrared analyses have been carried out on the acetamido acids rather than on the free amino-acids.

The applicability of an infrared analysis was first tested on a number of synthetic mixtures of acetyl-L-leucine and acetyl-L-isoleucine. The method was then extended to the examination of the leucine isomers obtained from various control mixtures, and finally leucine fractions from the hydrolysates of various proteins were studied.

ANALYTICAL PROCEDURE

Chemical

The leucine isomers were isolated by the method of Martin & Synge (1941). In the early stages of this work the procedure was to isolate the isomers on the quantitative column, using *c.* 25 mg. of protein. For a total leucine content of 10% this entailed the working up of some eight fractions. Now the R_F values of acetyl leucine and acetyl isoleucine are identical so that as long as the isolated samples are not submitted to recrystallization there is no need to attempt their quantitative isolation. Accordingly, the later samples were prepared qualitatively using 100–200 mg. of protein, the aim being to obtain about 20 mg. of mixed acetamido acids.

Procedure. The hydrolysate, equivalent to 100–200 mg. of protein, was acetylated in the usual manner (Gordon *et al.* 1943) using *c.* 20 ml. 6N-NaOH and 5·0 ml. acetic anhydride. After acidification to pH 1 with 10N-H_2SO_4, the mixture was adsorbed on silica gel, transferred to a large column (4·0 cm. diameter) and extracted with 1 l. chloroform-17% butanol (Tristram, 1946). The acetamido acids were then prepared for analysis and the leucine isomers isolated by the procedure outlined by Tristram (1946). In order to eliminate any acetylphenylalanine and acetylvaline, only the centre portion of the leucine band was collected. It was freed from colouring matter and indicator by passing it through water-saturated silica gel using *cyclo*hexane-5% propanol. The solvent was then removed and the acetamido acids allowed to crystallize in a vacuum desiccator.

Physical

Preparation of specimens for analysis. For accurate analytical work using infrared spectra, the sample should be either in the gaseous or liquid state, although solids which can be obtained as thin plates or films of which the thicknesses can be accurately determined are acceptable. Solvents having no intense absorption over the spectral regions investigated are very scarce in infrared work, and none of these was suitable for this problem. Estimations were accordingly made on the solid material suspended as a paste in liquid paraffin (Nujol). The acetamido acids were finely ground in a mortar and well mixed with an approximately equal quantity of Nujol until a smooth homogeneous paste was obtained. This paste was then pressed into a thin layer (approx. 30 μ) between two rocksalt plates and placed in the spectrometer beam. With this technique, the total amount of acetamido acids suspended in the radiation beam is not accurately known. The method accordingly leads to estimates of the relative rather than the absolute concentrations of the two isomers.

Quantities of materials used. The amount of material used in the analyses was limited by the yield of leucines obtained from the chromatographic separation; experiments were originally made on about 13–15 mg. of material, but later, smaller quantities, *c.* 7–10 mg., were found to be satisfactory for an assay if the sample was well dried and crystalline. The economy was principally achieved by having a suspension layer of very small area, placed close to the entrance slit of the spectrometer; a further reduction in quantity could probably be obtained by bringing the incident beam of the spectrometer to a subsidiary focus of small area. The concentration of the acetamido acid in the suspensions was such that the relevant absorptions were near the optimal percentage for accurate estimation.

Spectroscopic details. The earlier experimental work was done on a Hilger D. 88 spectrometer equipped with a rocksalt prism. The later work, on which all the data in this paper are based, was performed on a double-beam spectrometer (Hilger D. 209) with automatic pen recording (Sutherland & Thompson, 1945). At 700 cm.$^{-1}$, the rocksalt prism begins to absorb radiation, and the small amount of energy available makes accurate quantitative work difficult; accordingly we used a potassium bromide prism, which transmits to much lower frequencies in the infrared.

The resolving power of the D. 88 spectrometer is comparable with that of the D. 209 spectrometer at 700 cm.$^{-1}$ when the latter is equipped with a KBr prism. Recording of both spectrometers was automatic, and the spectral range 760–700 cm.$^{-1}$ was scanned several times for each specimen, to check reproducibility. In this way errors in the shape of the bands and the exact height and location of the maxima could be minimized.

Choice of bands for analysis. The infrared absorption spectra of acetyl-L-leucine, acetyl-L-isoleucine, acetyl-DL-leucine and acetyl-DL-isoleucine are shown in Fig. 1, in the region 1800–650 cm.$^{-1}$. The absorption bands are listed in Table 1. Although the spectra of the laevo compounds differ in many respects, it is advisable to choose two bands for analytical work which lie fairly close together. This is because these bands can then be examined under identical spectroscopic conditions (e.g. slit width).

It is clear from the spectra (Fig. 1) that the band of acetyl-L-leucine at 714 cm.$^{-1}$ and that of acetyl-L-isoleucine at 736 cm.$^{-1}$ satisfy this condition, and are yet sufficiently separated to be well resolved by the spectrometer. These bands do in fact overlap to a slight extent so that the intensities of absorption at 736 and 714 cm.$^{-1}$ are not entirely due to

acetyl-L-isoleucine and acetyl-L-leucine respectively. However, this can be taken into account in the calculations.

that no appreciable amount of racemization had occurred. The absorption spectra of the DL-acetamido acids are seen (Fig. 1 and Table 1) to be some-

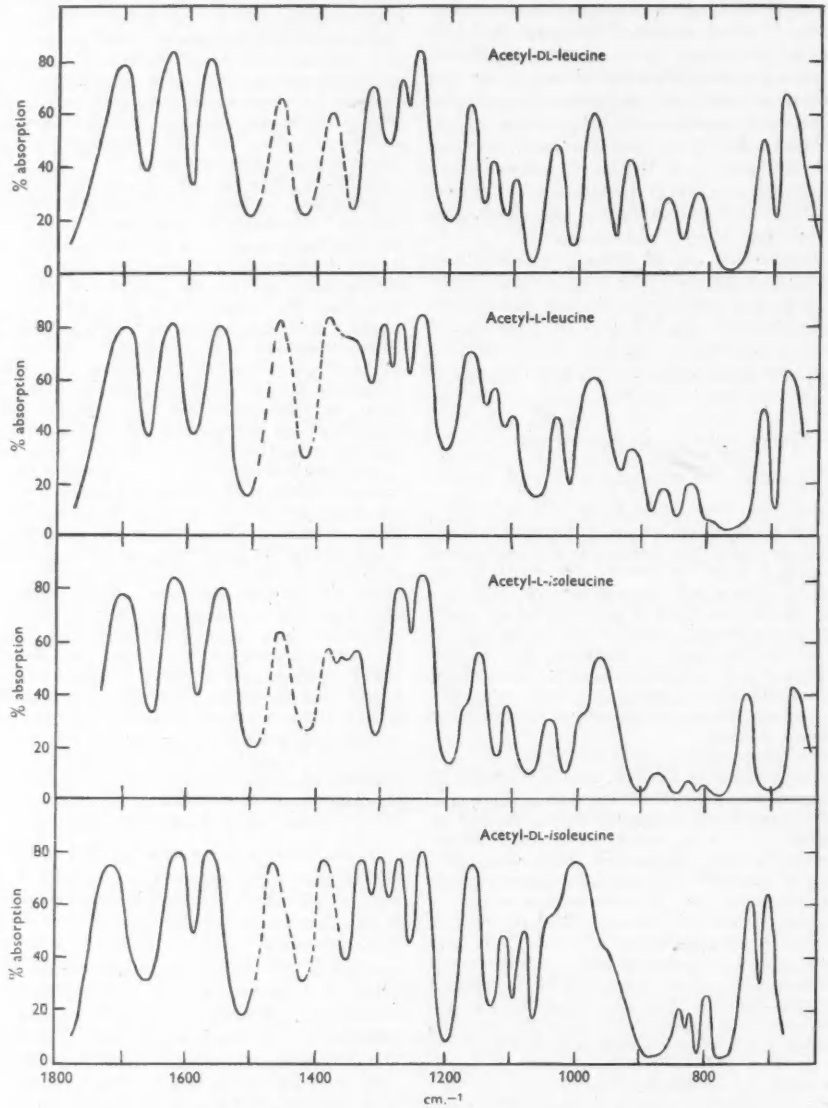

Fig. 1. Infrared absorption spectra of acetylleucines, 1800–650 cm.$^{-1}$. (Broken lines indicate Nujol absorption.)

The effects of racemization. The estimations were made on the assumption that the acetamido acids were present entirely in the laevo configuration and what different from those of the corresponding laevo compounds. This effect had been shown earlier by Wright (1937, 1939) for many amino-acids. The

presence of an appreciable proportion of racemized material in the chromatographic fraction may therefore render the estimation inaccurate. Acetyl-DL-leucine has an absorption peak at 714 cm.$^{-1}$ with approximately the same extinction coefficient as that of acetyl-L-leucine, but acetyl-DL-isoleucine gives rise to a broad absorption band centred at 726 cm.$^{-1}$ which would contribute to the absorptions measured at 736 and 714 cm.$^{-1}$. The spectra of corresponding L- and DL-leucines are not sufficiently different in the region 3600–750 cm.$^{-1}$ to enable a convenient check to be made on the presence of low proportions of DL-isomers in the fractions, but it is believed that the proportion of racemates in the specimens assayed is quite low.

Table 1. *Absorption maxima of the acetylleucines 1800–650 cm.$^{-1}$*

Acetyl-L-leucine	Acetyl-DL-leucine	Acetyl-L-isoleucine	Acetyl-DL-isoleucine
1695	1703	1703	1720
1624	1625	1622	1608
1554	1567	1547	1566
(1460) Nujol	(1460) Nujol	(1460) Nujol	(1460) Nujol
(1380) Nujol	(1380) Nujol	(1380) Nujol	(1380) Nujol
1347	1316	1364	1329
1298	1271	1339	1298
1276	1247	1270	1270
1244	1166	1238	1232
1164	1131	1170	1157
1127	1099	1150	1108
1103	1035	1105	1072
1034	976	1043	1042
974	920	993	996
917	864	965	835
863	815	876	820
822	714	823	793
796	681	803	726
714	—	736	701
678	—	665	—

Corrections for spurious and scattered radiation. It is important in quantitative analytical work in the infrared region to make allowance for spurious radiation. This is usually of higher frequency than the spectral range under examination, and may therefore have an appreciable magnitude in comparison with the energy being estimated. This is particularly important in the present case where the spectral region 750–700 cm.$^{-1}$ is so far removed from the region of maximum energy of the Nernst source. Spurious radiation of frequency greater than about 900 cm.$^{-1}$ is allowed for by placing a fluorite plate in the spectrometer beam to measure the effective zero energy at 750–700 cm.$^{-1}$ (fluorite is opaque for frequencies less than 900 cm.$^{-1}$). This zero setting was found to be appreciably different from that obtained when the beam was cut off with a metal shutter, showing that a small proportion of high-frequency radiation greater than 900 cm.$^{-1}$ was being received by the detector when the 750–700 cm.$^{-1}$ region was being scanned. That the amount of radiation scattered by the solid particles in suspension is probably very small in this region of the spectrum has been shown by comparing the degree of transmission of the sample, at a frequency well removed from an absorption band, with the degree of transmission of a layer of Nujol between rocksalt plates at that same frequency. Scattering effects have therefore been neglected.

Methods of calculating concentrations

We assume the validity of Beer's law, viz.

$$I_\nu = I_{0\nu} e^{-kc}, \tag{1 a}$$

relating the intensity of the transmitted radiation I_ν, the incident intensity $I_{0\nu}$ of the same frequency, the number of gram molecules c placed in the spectrometer beam and the molecular extinction coefficient k. We further assume that Beer's law may be extended additively for mixtures of two components,

$$I_\nu = I_{0\nu} e^{-(kc + k'c')}. \tag{1 b}$$

The incident intensity $I_{0\nu}$ is measured experimentally as the intensity of radiation of frequency ν, transmitted through the absorption cell containing Nujol only. This procedure allows for radiation reflected from the cell faces, and radiation absorbed and scattered by the cell and the Nujol.

Let c = number of gram molecules of acetyl-L-leucine, c' = number of gram molecules of acetyl-L-isoleucine, I_{01} = the incident intensity at 714 cm.$^{-1}$, I_1 = transmitted intensity at 714 cm.$^{-1}$, I_{02} = incident intensity at 736 cm.$^{-1}$, I_2 = transmitted intensity at 736 cm.$^{-1}$, k_1 = molecular extinction coefficient of acetyl-L-leucine at 714 cm.$^{-1}$, k_2 = molecular extinction coefficient of acetyl-L-leucine at 736 cm.$^{-1}$, k_1' = molecular extinction coefficient of acetyl-L-isoleucine at 714 cm.$^{-1}$, k_2' = molecular extinction coefficient of acetyl-L-isoleucine at 736 cm.$^{-1}$. Then, from Beer's law,

$$\log_e \frac{I_{01}}{I_1} = k_1 c + k_1' c', \tag{2 a}$$

$$\log_e \frac{I_{02}}{I_2} = k_2 c + k_2' c'. \tag{2 b}$$

Let d_1 = optical density at 714 cm.$^{-1}$ $= \log_e \frac{I_{01}}{I_1}$, (3 a)

and d_2 = optical density at 736 cm.$^{-1}$ $= \log_e \frac{I_{02}}{I_2}$. (3 b)

Then, solving (2 a) and (2 b) for c and c', we have

$$c = \frac{d_1 k_2' - d_2 k_1'}{k_1 k_2' - k_2 k_1'}, \tag{4 a}$$

$$c' = \frac{d_1 k_2 - d_2 k_1}{k_2 k_1' - k_1 k_2'}, \tag{4 b}$$

or

$$\frac{c'}{c} = \frac{d_1 k_2 - d_2 k_1}{d_2 k_1' - d_1 k_2'}. \tag{5}$$

The terms of the right-hand side of equation (5) are homogeneous in d, so that common logarithms may be used throughout. We may rewrite this

$$\frac{c'}{c} = K \frac{d_2 - d_1 k_a}{d_1 - d_2 k_b}, \tag{6}$$

where K, k_a, k_b, are constants related to the molecular extinction coefficients, and which are easily determined from the experimental data, i.e.

$$K=\frac{k_1}{k_2'}, \quad k_a=\frac{k_2}{k_1}, \quad k_b=\frac{k_1'}{k_2'}.$$

This equation enables the ratio of the quantities of the two isomers to be calculated in a manner independent of the total quantity of material present.

maxima), and the conditions studied under which the assumptions of Beer's law stated in the previous section, are justified. The relevant absorption bands of some of these mixtures are shown in Fig. 2.

It was found that, for a given mixture, the value of $(d_2-d_1k_a)/(d_1-d_2k_a)$ is constant, within the limits of experimental error, for a moderate range of d_1 and d_2. For path lengths giving greater than about

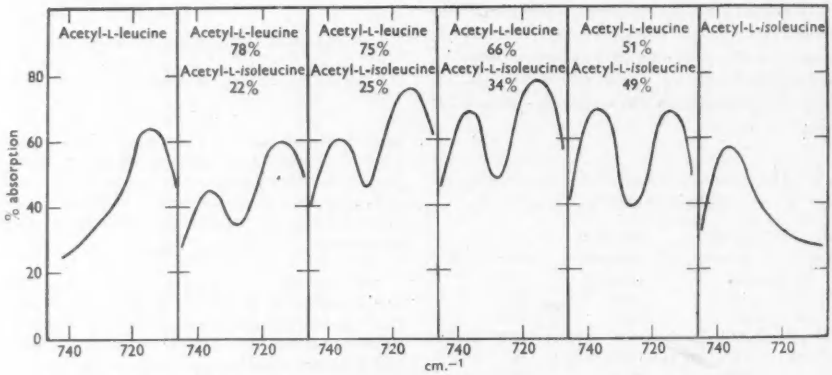

Fig. 2. Absorption spectra of some mixtures of known composition of acetyl-L-leucine and acetyl-L-isoleucine, 745–710 cm.$^{-1}$.

Evaluation of K, k_a, k_b. k_a, k_b are evaluated from the absorption curves for pure acetyl-L-leucine and acetyl-L-isoleucine. For the pure leucine isomer, $c'=0$, and from equation (6), $k_a=\left[\frac{d_2}{d_1}\right]_0$, the subscript 0 indicating that d_1 and d_2 are the optical densities for the pure leucine isomer.

Similarly, for the pure isoleucine isomer, $k_b=\left[\frac{d_1}{d_2}\right]_0$.

If a series of isomeric mixtures of known proportions is examined and the function $\frac{d_2-d_1k_a}{d_1-d_2k_b}$ plotted graphically against c'/c, a straight line should be obtained, the value of the slope being the constant K. Having determined the line and relation between c'/c and the function of the optical densities d_1 and d_2, the value of c'/c may be obtained directly from the graph for the value of

$$(d_2-d_1k_a)/(d_1-d_2k_b)$$

given by any mixture of unknown composition.

RESULTS

The calibration data

Synthetic mixtures of the pure crystalline acetyl-L-leucines were examined over a range varying from 0 to 50 % isoleucine; mixtures containing more than 50 % isoleucine were not examined, analytical data for the proteins indicating that isoleucine is invariably found in smaller amounts than leucine. Each synthetic mixture was examined at different path lengths (giving different absorptions at the 85 % absorption, the intensity of absorption of the weaker band is apparently enhanced, giving high values of c'/c. When the bands are weak (the stronger band being less than about 40 % absorption), the intensities cannot be measured with sufficient precision to enable c'/c to be determined accurately. We have therefore examined samples such that the absorption intensities of the stronger band lie between about 40 and 80 %, both in the calibration and in the estimation of mixtures of unknown composition.

The evaluation of the calibration data is set out in Table 2 (*a*). Substituting the values $k_a=0{\cdot}36$ and $k_b=0{\cdot}40$, from this table, into equation (6),

$$c'/c=K(d_2-0{\cdot}36d_1)/(d_1-0{\cdot}40d_2). \qquad (7)$$

But $d_1=\log_{10}100/I_1$ and $d_2=\log_{10}100/I_2$, so that

$$c'/c=K\,(1{\cdot}28-\log_{10}I_2+0{\cdot}36\log_{10}I_1)/(1{\cdot}20-\log_{10}I_1+0{\cdot}40\log_{10}I_2). \qquad (7a)$$

Equation (7*a*) is the form of equation (7) which is used in the numerical calculations.

The absorption data for mixtures of known composition are given in Table 2 (*b*); the values of $(d_2-0{\cdot}36d_1)/(d_1-0{\cdot}40d_2)$ calculated from these give a straight line when plotted against the corresponding values of c'/c (Fig. 3); this is taken as the working curve in the analysis of unknown mixtures. When $c'=0$ (pure leucine), $(d_2-0{\cdot}36d_1)/(d_1-0{\cdot}40d_2)$ is equal to zero, so that the curve passes through the origin.

Table 2. *Calibration data*

(*a*) Evaluation of k_a and k_b

	Percentage transmission at 714 cm.$^{-1}$ (I_1)	Percentage transmission at 736 cm.$^{-1}$ (I_2)	k_a	k_b
Acetyl-L-leucine	38·5	71·0	0·358	—
	36·0	69·5	0·357	—
Acetyl-L-isoleucine	71·0	43·0	—	0·406
	75·0	48·5	—	0·398

Mean value of $k_b = 0{\cdot}36$. Mean value of $k_b = 0{\cdot}40$.

(*b*) Transmission of synthetic mixtures

Percentage ratios leucine : isoleucine	c'/c	Transmissions I_1	Transmissions I_2	$\frac{d_2 - 0{\cdot}36\,d_1}{d_1 - 0{\cdot}40\,d_2}$	Mean value
51 : 49	0·96	31·5	30·5	1·14	1·15
		56·5	55·0	1·18	
		60·5	59·5	1·15	
66 : 34	0·515	19·0	28·0	0·59	0·59
		20·5	29·5	0·59	
		21·5	31·0	0·58	
		42·0	52·5	0·56	
75 : 25	0·33	24·0	39·0	0·41	0·40
		61·5	73·0	0·39	
		66·0	78·0	(0·31)	
78 : 22	0·28	40·0	55·0	0·39	0·38
		61·0	73·0	0·36	
		60·0	71·5	0·40	

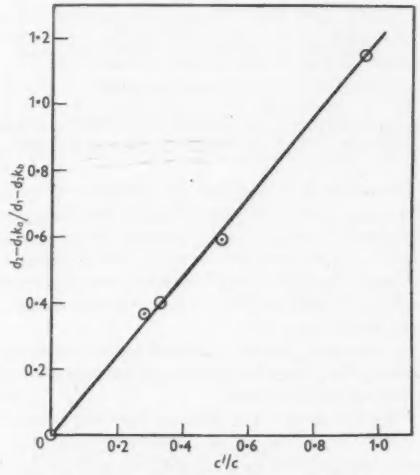

Fig. 3. Calibration curve for leucine-isoleucine analysis.

The calibration data in Table 2 are given in detail to enable an estimate to be made of the 'spread' of the results obtained by this method of analysis.

Control mixtures

Two control experiments were performed to test the validity of the chemical procedure.

(i) Known proportions of the acetyl-L-leucines were evaporated together from solution in the same way as the leucine fractions from the chromatograph column. The mixture had not been treated chromatographically. The results of the spectroscopic analysis are shown in Table 3. The agreement between the analytical values and the original composition indicates that the spectra of the isomers after these have been crystallized together from solution are still additive.

Table 3. *Estimation of isoleucine in a control mixture of leucines*

Isoleucine as % total leucine isomers	
Present in original mixture	Estimated spectroscopically
32·2	30·3

(ii) A mixture of acetamido acids, in the proportions in which they occur in an insulin analysis (Tristram, 1946), and having an isoleucine/leucine ratio of 1 : 4, was passed through the chromatograph column. The leucine fraction was isolated and the isomer ratio estimated spectroscopically (Table 4).

Table 4. *Control experiment on 'synthetic insulin hydrolysate'*

Isoleucine as % total leucine isomers	
Present in original 'hydrolysate'	Estimated spectroscopically
19·8	18·7, 18·7, 17·5

The estimated ratios agree within 2% with the ratio originally present, the proportion of isoleucine estimated being low. This control experiment indicates that the leucine isomers are not differentiated in the chromatograph column.

Proteins

The isoleucine/leucine ratio has been estimated in the hydrolysates of three proteins, casein, edestin and insulin (two samples from independent hydrolysates). The ratios are given in Table 5. Several assays were made in each sample at varying optical densities; consistent values of the isoleucine/leucine ratio were obtained from assays having absorptions near the optimal value for accurate measurements. The spectra of these samples were examined carefully in the range 1800–700 cm.$^{-1}$ and compared with those of similar synthetic mixtures of acetyl leucine and isoleucine. Agreement throughout the spectral range was good and no evidence of extraneous bands, arising from impurities, was found. The spectra of acetyl-L-valine, and the acetyl-DL-leucines were particularly examined in this connexion.

Table 5. *The isoleucine/leucine ratio as determined spectroscopically in some proteins*

Protein	c'/c	Isoleucine as % of total leucines	
Casein	0·46	32	
Edestin	0·24	19	Two assays
	0·20	17	
Insulin (first sample)	0·205	17	Two assays
	0·19	16	
Insulin (second sample)	0·23	18·5	Three assays (mean value 15–18%)
	0·17	14·5	
	0·18	15·3	

DISCUSSION

The reliability and accuracy of the method

The assumption made in these calculations is that the relative absorption intensities of the isomers at two fixed frequencies can be expressed by Beer's law, in a form which is independent of the total amount of absorbing material. The two isomers are examined together as a physical mixture of the solids. The possibility that intermolecular interaction (such as might arise by crystallizing the isomers together) could invalidate the use of Beer's law has been shown to be absent. The validity of these assumptions is confirmed by the experimental data from the synthetic mixtures (Fig. 3), which show a constant value for K, the slope of the curve.

Reproducibility of the percentage absorptions which are measured is checked by scanning each mixture twice or more; the percentage absorptions are then considered accurate to $\pm 0{\cdot}5\%$ of absorption. The corresponding error in the percentage of isoleucine in the mixture is plotted in Fig. 4 against the percentage of isoleucine. It is thus seen, for example, that when the percentage of isoleucine is near 19%, this figure is expressed as $(19 \pm 1{\cdot}5)\%$. In order not to overstate our accuracy, the errors in measurement of the two bands have been assumed to be cumulative. The independence of total concentration is ascertained by examining each sample at two or more optical densities. Reasonably good agreement is obtained when the absorption of the stronger component is within the range 40–80%, and when the weaker band is not too feeble to be measured accurately.

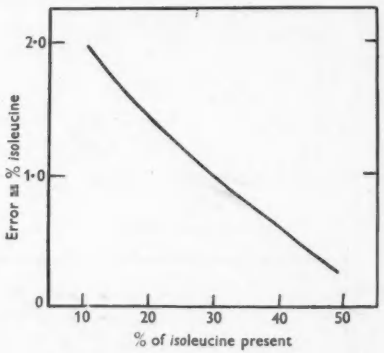

Fig. 4. Error curve for isoleucine estimation, assuming errors of $\pm 0{\cdot}5\%$ in measuring absorption bands.

The method is capable of refinements and by adopting a standard procedure of working, with adequate controls, it can probably be made a very reliable method of amino-acid analysis. Results are only reproducible if identical spectroscopic working conditions both for the calibration and assays are maintained.

As discussed earlier, racemization would cause errors in the present spectroscopic estimations of the isoleucine/leucine ratio.

Whether or not the leucine fraction is fully recovered from the chromatograph column, the spectroscopic method of analysis should yield a reliable value of the isoleucine/leucine ratio.

Comparison of the method with microbiological assay

The greatest difference between the spectroscopic method and that of microbiological assay is the relatively large amount of material needed for the former. For a spectroscopic analysis, we require at present 5–10 mg., compared with the few μg. required for a microbiological assay. Moreover, the

spectroscopic method, as used at present, gives the relative concentrations of the isomers, whereas absolute concentrations of the amino-acids are given by microbiological assay.

The advantage of the spectroscopic method over microbiological assay is that a highly characteristic physical property of the molecule (its vibration spectrum) is being used. The microbiological method is subject to doubts, particularly regarding lack of specificity of the organism, and interference of other constituents in a mixture. The microbiological method is rendered inaccurate by racemization, the D-form in general not being estimated (see Dunn, Camien, Shankman & Block, 1947), and the assay therefore being low in such a case. The effect of racemization in spectroscopic estimation has already been discussed, but there is little doubt that a more refined technique could make due allowance for this. We would stress that the present investigations have been of an exploratory nature, and that the method is capable of greater accuracy with a carefully standardized routine.

Discussion of analysis

The mean values of percentages of isoleucine in total leucine fractions shown in Table 5 are considered reliable to $\pm 1{\cdot}5\,\%$ (see Fig. 4), although the control experiments suggest that there may be a tendency for the present results to be systematically low. The comparison with microbiological values is given in Table 6. It will be observed that the spectroscopic values for casein and insulin are close to those previously obtained by microbiological assay, but the isoleucine proportion is slightly low in both cases.

Table 6. *Comparison of spectroscopic values of the isoleucine/leucine distribution with microbiological values*

	Percentage of isoleucine in total leucine fraction	
	Spectroscopic	Microbiological assay
Casein	32	38·5*, 39·5†, 39·2‡, 36·2§
Edestin	18	45·5‖
Insulin	15–18	17·8¶

* Hier, Graham, Friedes & Klein (1945).
† Kuiken, Norman, Lyman, Hale & Blotter (1943).
‡ Dunn, Camien, Rockland, Shankman & Goldberg (1944).
§ Stokes, Gunness, Dwyer & Caswell (1945).
‖ Smith, Greene & Bartner (1946); Smith & Greene (1947).
¶ Brand (1946).

An earlier spectroscopic value for the ratio of the leucine isomers, which was given in a publication by Tristram (1946), was in error, due to imperfect calibration data obtained in the preliminary stages of the investigations on insulin.

The value of comparing one method with another based upon different principles is indicated very strikingly in the case of the ratio of the leucine isomers of edestin. Smith *et al.* (1946) and Smith & Greene (1947) reported that the leucine (*Lactobacillus arabinosus*) and isoleucine (*Streptococcus faecalis*) contents of edestin were 4·25 and 3·55 % respectively (i.e. 54·5 % of leucine). Infrared analysis suggests that the distribution is nearer 80 % leucine (Table 5).

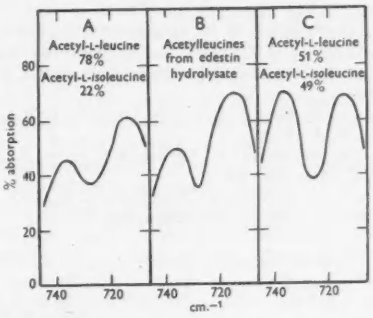

Fig. 5. Comparison of spectra of acetylleucines from edestin hydrolysate with mixtures of known composition.

It has been shown that the chromatographic separation of the acetylleucines fraction does not effect any concentration of isoleucine, and whatever the recovery of the total leucine isomers, the ratio is unaltered. Two of the calibration mixtures (Table 2*b*), containing respectively 51 and 78 % leucine, are near to the distributions suggested respectively by microbiological and spectroscopic methods, and the absorption curves of these mixtures are compared in Fig. 5 with that obtained from the leucines fraction of edestin.

SUMMARY

1. The infrared absorption spectra of leucine and isoleucine and of their acetyl derivatives have been examined in the range 1800–650 cm.$^{-1}$. The spectra of each pair of isomers show marked differences which make it easy to distinguish the isomers.

2. A spectroscopic method of analyzing isoleucine/leucine mixtures has been investigated (using as indicators characteristic bands near 700 cm.$^{-1}$ of the acetyl derivatives) and tested on synthetic mixtures of the L-isomers.

3. The applicability of the spectroscopic method of analysis for estimating the isoleucine/leucine ratio in the leucine fraction of protein hydrolysates has been tested by control experiments on mixtures of leucine and isoleucine subjected to the treatment employed in protein analyses.

4. The study of the method in the determination of the isoleucine/leucine ratio in the leucine fraction from the hydrolysates of casein, insulin and edestin gave results which agree with those obtained by microbiological methods except in the case of edestin where there is a very large discrepancy.

We are grateful to Prof. A. C. Chibnall, at whose instigation this investigation was undertaken, for his interest and helpful advice. We are indebted to the Medical Research Council (S.E.D.) and to the Agricultural Research Council (G.R.T.) for grants during the tenures of which this investigation was undertaken.

REFERENCES

Brand, E. (1946). *Ann. N.Y. Acad. Sci.* **47**, 187.
Dunn, M. S., Camien, M. N., Shankman, S. & Block, H. (1947). *J. biol. Chem.* **168**, 43.
Dunn, M. S., Camien, M. N., Rockland, L. B., Shankman, S. & Goldberg, S. C. (1944). *J. biol. Chem.* **155**, 591.
Edsall, J. T. (1938). *Cold Spr. Harb. Symp. quant. Biol.* **6**, 40.
Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1943). *Biochem. J.* **37**, 79.
Hier, S. W., Graham, C. E., Friedes, R. & Klein, D. (1945). *J. biol. Chem.* **161**, 705.
Kuiken, K. A., Norman, W. H., Lyman, C. M., Hale, F. & Blotter, L. (1943). *J. biol. Chem.* **151**, 615.
Martin, A. J. P. & Synge, R. L. M. (1941). *Biochem. J.* **35**, 1358.
Smith, E. L. & Greene, R. D. (1947). *J. biol. Chem.* **167**, 833.
Smith, E. L., Greene, R. D. & Bartner, E. (1946). *J. biol. Chem.* **164**, 159.
Stokes, J. L., Gunness, M., Dwyer, L. M. & Caswell, M. C. (1945). *J. biol. Chem.* **160**, 35.
Sutherland, G. B. B. M. & Thompson, H. W. (1945). *Trans. Faraday Soc.* **41**, 174.
Tristram, G. R. (1946). *Biochem. J.* **40**, 721.
Wright, N. (1937). *J. biol. Chem.* **120**, 641.
Wright, N. (1939). *J. biol. Chem.* **127**, 137.

Studies on Vitamin A

5. THE PREPARATION OF RETINENE$_1$—VITAMIN A ALDEHYDE

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The state of knowledge concerning photoreception and the role of rhodopsin (visual purple) has been surveyed recently (Granit, 1947) so that only a brief introduction is necessary here.

Wald (1935–6) found that dark-adapted retinas, which are pinkish purple in colour owing to the presence of relatively large amounts of rhodopsin, turn yellow on illumination. Light petroleum will extract from retinas exposed to light a material which Wald called *retinene*. The petroleum solution, freed from solid matter by centrifuging, shows an ultraviolet-absorption maximum near 365 mμ., displaced to 385 mμ. when chloroform is the solvent. When the antimony trichloride reagent, used in vitamin A studies, is added to a chloroform solution of retinene, a blue colour is produced. The blue solution exhibits a well-defined absorption band with a maximum at 664 mμ. Wald postulated for scotopic vision a cyclic process into which entered vitamin A, retinene and visual purple.

In later studies, Wald (1937, 1938–9 *a*, *b*) found that a similar extraction procedure applied to retinas of fresh-water fish, in which rhodopsin is largely replaced by porphyropsin (visual violet), yielded retinene$_2$. This substance is similar to retinene$_1$ but its absorption spectrum is slightly displaced in the direction of longer wave-lengths, as also is the characteristic absorption maximum of the colour test. The discovery that vitamin A$_2$ (Edisbury, Morton & Simpkins, 1937; Lederer & Rosanova, 1937) tends to take the place of vitamin A in fresh-water fish as compared with sea fish and mammals indicated that retinene$_2$ bears the same relationship to vitamin A$_2$ as retinene$_1$ to vitamin A. This is not surprising, as there was already ample evidence of a connexion between vitamin A deficiency and night blindness associated with retarded formation of rhodopsin (visual purple) in the dark-adaptation process.

The discovery of the retinenes was a notable advance, but paucity of material has delayed its exploitation. The amounts obtainable from eyes are too minute to permit orthodox constitutional investigations and for nearly a decade the substances have remained known only by qualitative spectroscopic characteristics quoted in Table 1.

Table 1. *Absorption maxima of substances concerned in visual processes*

Substance	$\lambda_{max.}$ (mμ.)	Solvent	$SbCl_3$ colour test (in chloroform) $\lambda_{max.}$ (mμ.)
Vitamin A	326	*cyclo*Hexane	617 inflexion 582
Vitamin A$_2$	345–350	*cyclo*Hexane	693
Rhodopsin (visual purple)	502	1% aqueous digitonin	
Porphyropsin	520–530	1% aqueous digitonin	
Retinene$_1$	369·5	Light petroleum	664
	389	Chloroform	
Retinene$_2$	390	Petrol	>705
	405	Chloroform	

The conclusion drawn by Wald, that retinene$_1$ is a carotenoid and rhodopsin a conjugated protein with retinene as its prosthetic group, has been much quoted, but it is rather insecurely based. Some writers use the term 'carotenoid' as a class designation for highly coloured C_{40} polyenes including those containing hydroxyl and carboxyl groups. Others include in the class C_{20} polyenes such as vitamin A, which are almost colourless. It is arguable that this loose use of the word carotenoid is permissible, but the suggestion that retinene is itself the prosthetic grouping of visual purple is difficult to reconcile with the displacement of the 369 mμ. maximum of retinene to 502 mμ. in visual purple.

No questions of terminology or tentative interpretation can, however, weaken Wald's demonstration that the vitamins A and the visual pigments are somehow linked through the retinenes. From a chemical standpoint, the preparation of the pure retinenes, the elucidation of their structures, and the changes they can be made to undergo are major objectives in the study of visual processes.

Morton (1944), in a survey of the problem based primarily on the interpretation of absorption spectra, came to the conclusion that retinene$_1$ must be the aldehyde of vitamin A. By far the most plausible explanation of the 369 mμ. absorption maximum of retinene$_1$ was the replacement of CH_2OH in vitamin A by CHO, as this would give six conjugated double bonds instead of five, and so account for the displacement from 326 to 369 mμ. Oxidation at any other point in the vitamin A molecule could not fail to displace the absorption in the opposite direction. Similar arguments were valid for the retinene$_2$-vitamin A$_2$ system. The task of preparing such an aldehyde seemed formidable because of the five conjugated double bonds present in the vitamin A molecule. It was not easy to see how ordinary oxidative processes could be restricted to the terminal CH_2OH group and a study of the literature showed that in the Oppenauer reaction any aldehyde formed would tend to undergo further change. Considerations of that kind did not, however, dispose of the matter.

Direct oxidation of vitamin A might be expected to result in simultaneous attack upon the terminal CH_2OH group and upon the double bonds, yielding a variety of products, but the possibility that a little vitamin A aldehyde might be formed, which would escape further attack, seemed worthy of investigation, especially as chromatographic adsorption gave some promise of a separation of the mixture. Morton & Goodwin (1944), by shaking vitamin A concentrates dissolved in light petroleum with dilute aqueous $KMnO_4$ in the presence of a little H_2SO_4 and chromatographic separation of the products, always obtained some fractions which showed an absorption band at 365–370 mμ. in saturated hydrocarbon solvents and at 385 mμ. in chloroform. In the latter solvent the solute gave a 664 mμ. maximum with the antimony trichloride reagent. The yields of spectroscopically homogeneous material were small, but there could be no doubt that retinene was an oxidation product of vitamin A, and that it was very probably the simple aldehyde.

The suggestion that retinene was vitamin A aldehyde was soon confirmed by Hunter & Hawkins (1944), who obtained from vitamin A by the Oppenauer reaction a material showing two absorption maxima at 350 and 368 mμ. respectively, and giving a colour-test maximum at 657 mμ. It also yielded a 2:4-dinitrophenylhydrazone, m.p. 207–209°, and was converted to vitamin A by the Pondorff reduction. Hunter & Hawkins were kind enough to allow two of us to test their product. The difference between 657 and 664 mμ. turned out to be a matter of spectroscopic technique only, but the presence of an ultraviolet maximum at 350 mμ. was not compatible with homogeneity. Their material, though rich in retinene, was clearly impure.

A recent preliminary paper by van Dorp & Arens (1947) describes an alternative synthesis of vitamin A aldehyde in impure form.

Orientating experiments in which the present authors were assisted by S. Roberts showed that potassium permanganate was the most suitable oxidizing agent and that variables such as temperature, concentration of reactants, relative volumes of phases, emulsification, etc. were of importance. Some retinene was always obtained, but yields were generally poor and not very reproducible. The one consistent tendency seemed to be an improved yield when hydrated manganese dioxide was formed, i.e. when the sulphuric acid used was insufficient to give a clear solution. An apparatus was then made in which small droplets of a solution of vitamin A in light petroleum were allowed to ascend a tall column of permanganate solution. Reaction between vitamin A and permanganate was very sluggish and it seemed possible that retinene could not be formed until some manganese dioxide was present.

Some years before Wald discovered retinene, one of us in collaboration with Sir Jack Drummond had studied the fate of vitamin A concentrates left to stand over various solids. Nothing came of the work at the time, but re-examination of the working notebooks showed that a colour-test maximum had been seen at 664 mμ. in a solution which had been in contact with manganese dioxide. A feeble band in the same position had been seen in tests with residues from the purification of carotene. At that time the mechanism of the antimony trichloride colour test was even more uncertain than it is now, and a maximum at 664 mμ. conveyed no more than a hint of some unknown impurity or artefact. When, however, the 664 mμ. band became a 'label' for retinene, the old observations slipped into a pattern. It was therefore decided to investigate the adsorption of vitamin A from inert solvents on solid oxidizing agents, in the hope of limiting the attack to the primary-alcohol grouping.

The outcome has been a smooth conversion to retinene at room temperature when vitamin A in light petroleum was allowed to stand over manganese dioxide.

EXPERIMENTAL

Materials and apparatus

Vitamin A. Two types of preparation were used:

(1) A vitamin A alcohol concentrate, from the British Drug Houses Ltd., of $E^{1\%}_{1\,cm.}$ 325 mμ. 600 (*c.* 1,000,000 i.u./g.).

(2) A vitamin concentrate prepared in the laboratory from a rich halibut-liver oil. The non-saponifiable matter was dissolved in methanol and most of the sterols were crystallized out at −35°. The filtrate was freed from solvent and the residue showed $E^{1\%}_{1\,cm.}$ 326 mμ. 900, indicating the presence of more than 50% vitamin A alcohol.

Manganese dioxide. Three types of material were used:

Sample A was the ordinary granular laboratory reagent.

Sample B was a much finer grade of the commercial product, but differed from *A* only in the size of the granules.

Sample C was prepared by mixing aqueous solutions of equivalent amounts of $MnSO_4$ and $KMnO_4$, filtering, washing until free from sulphate ions and drying on a porous plate in a desiccator.

Light petroleum. The ordinary material, b.p. 40–60°, was mainly used, but for some of the work a 60–80° fraction purified for spectroscopy was substituted.

Alumina. Small quantities of 'Brockman' activated alumina (Merck) were at first available. A material purchased from Savory and Moore Ltd. proved satisfactory, but the greater part of the work was carried out using Grade 0 activated alumina (Peter Spence and Co.) weakened by incorporation of water to the extent of 10% (w/w) just before preparing the column.

Antimony trichloride reagent. For most of the work a saturated solution of anhydrous antimony trichloride in B.P. chloroform was used, the solvent having been previously left to stand over $CaCl_2$ to remove ethanol.

Ultraviolet-absorption spectra were determined by means of the Beckman quartz photoelectric spectrophotometer and the colour-test observations were made visually using a Hilger-Nutting spectrophotometer.

Preparation of retinene

A convenient amount (0·2 g.) of vitamin A concentrate ($E^{1\%}_{1\,cm.}$ 325 mμ. 600) is dissolved in light petroleum (100 ml.) in a 250 ml. conical flask, and a calculated quantity of manganese dioxide (5 g. sample *A*) added. The weight of oxide to be used depends upon its state of subdivision, and is arrived at by a few small-scale preliminary experiments. The flask is tightly corked to prevent escape of solvent and is left in the dark at room temperature. The mixture is shaken occasionally (but this is not essential) and at the end of 6–10 days the transformation to retinene is almost complete. Longer periods of standing reduce the yield of retinene. The solution is now filtered and the manganese dioxide is washed once with light petroleum. Yields up to 80% calculated on the vitamin A used have been obtained. For example, in one experiment the product exhibited an almost symmetrical absorption curve with a maximum at 368 mμ. ($E^{1\%}_{1\,cm.}$ 390). With the antimony trichloride reagent a blue solution was obtained, $\lambda_{max.}$ 664 mμ. By applying the correction procedure of Morton & Stubbs (1946) the contribution attributable to unchanged vitamin A was $E^{1\%}_{1\,cm.}$ 325 mμ. 50, and that due to retinene was $E^{1\%}_{1\,cm.}$ 367 mμ. 382. The retinene is purified by chromatographic adsorption on slightly weakened alumina (*v.s.*), and fractions exhibiting $E^{1\%}_{1\,cm.}$ 365 mμ. 1250 (in light petroleum) are regularly obtainable.

As the maximum extinction coefficients of vitamin A and retinene do not differ greatly in light petroleum, the results imply 75% conversion and contamination of the product by about 10% of unchanged vitamin A.

The effect of agitation. A 0·5 g. portion of a concentrate, containing about 0·16 g. vitamin A alcohol, was dissolved in 250 ml. light petroleum; 100 ml. of the solution and 5 g. MnO_2 (sample *A*) were placed in a glass-stoppered bottle of 250 ml. capacity. The mixture was subjected to brisk but not violent agitation on a mechanical shaker for 8 hr. daily. A similar mixture was left to stand in the dark without shaking. After 6 days, measured portions of the two solutions were removed, and the blue solutions obtained with the antimony trichloride reagent were studied quantitatively using the wavelengths 617 and 664 mμ. characteristic respectively of vitamin A and retinene and afforded the following results:

	$E^{1\%}_{1\,cm.}$ at 617 mμ.	$E^{1\%}_{1\,cm.}$ at 664 mμ.
Without agitation	754	554
With agitation	768	710

Agitation thus resulted in an appreciably improved yield of retinene. The apparent increase in vitamin A in the second case was due to the fact that, although the retinene colour-test absorption band shows a sharp maximum near 664 mμ., there is also considerable absorption at 617 mμ. This becomes superimposed upon that from residual vitamin A, so that the intensity of absorption at 617 mμ. leads to an overestimate of the vitamin A present.

The effect of agitation is reproducible, but one further experiment requires to be mentioned. In this, a Waring Blendor was used to secure extremely vigorous agitation. Under normal conditions several hours are required for the appearance of the retinene spectrum, but in this case considerable amounts of retinene appeared within 1·5 hr. The heat generated by the very rapid stirring caused much evaporation of the light petroleum. The use of the Blendor was discontinued as being unnecessarily hazardous. For work on a larger scale, vigorous stirring in a suitably designed apparatus may well be worth while.

Rate of production of retinene. Many experiments on the time factor were carried out. The following is typical: 0·2 g. of concentrate (0·06 g. vitamin A) was dissolved in 100 ml. of purified light petroleum and the solution was left over manganese dioxide (5 g. sample *A*) in the dark. Sample portions were withdrawn at intervals, appropriately diluted, and examined spectrophotometrically. The curves shown in Figs. 1–4 exhibit a steady decrease in the intensity

of the vitamin A maximum with a corresponding increase in absorption at 369 mμ.

The reaction can also be followed spectrographically. Figs. 1–4 indicate how the absorption spectrum of a test solution gradually widens and moves towards longer wave lengths as retinene becomes the predominating solute. Applying the correction procedure (Morton & Stubbs, 1946) to these curves, the change in vitamin A concentration in the solution can be determined. Using data obtained for crystalline retinene a similar correction can be used to compute the corresponding retinene concentration. The sum of the two computed curves is not identical with the observed curve. The difference curve is wide and of indeterminate structure; its origin is unknown.

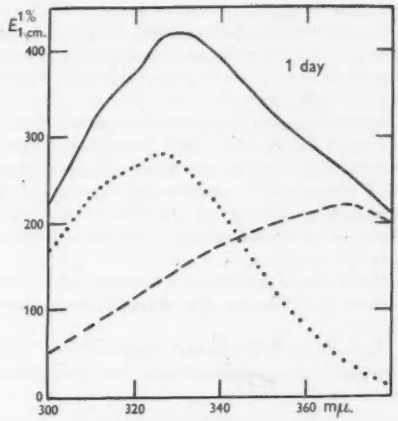


Fig. 1.

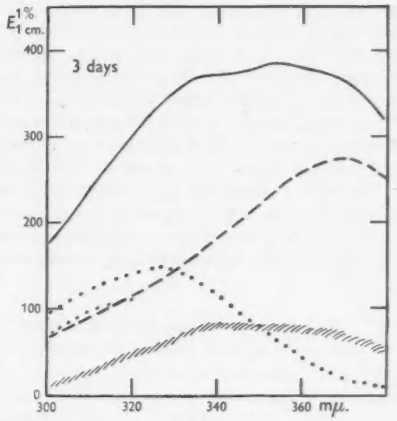


Fig. 2.

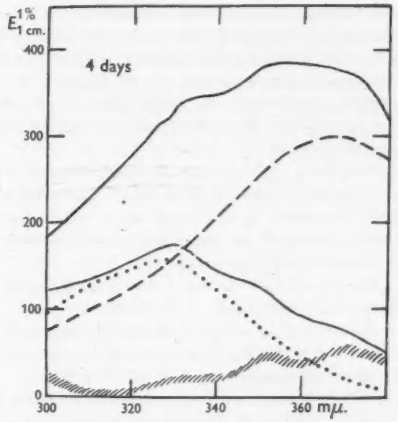


Fig. 3.

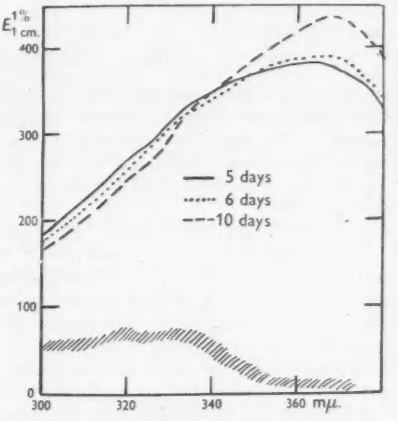


Fig. 4.

Figs. 1–4. Absorption curves obtained when a vitamin A concentrate is left over manganese dioxide in the dark (solvent, light petroleum) for periods of 1–10 days. The vitamin A band is gradually replaced by the absorption due to retinene$_1$ (measurements at intervals of 5 mμ. except around maxima where intervals were 0·5 mμ.). Observed absorption ——; vitamin A by correction procedure · · · · ·; retinene by correction procedure - - - - - -; residual absorption after correcting for vitamin A and retinene //////.

Relative amounts of vitamin A, manganese dioxide and petroleum. The amount of manganese dioxide required for the best yield is critical and depends upon two factors: (*a*) the state of subdivision of the

sample, and (*b*) the absolute amount of vitamin A in the reacting solution. Tests have been made with three different samples and good results have been obtained with each. It is, however, clear that the finer the state of subdivision, the smaller is the amount of oxide needed for the maximum yield of retinene. Thus with 1 g. of concentrate (0·3 g. vitamin) in 100 ml. of light petroleum, the best results were obtained with 25 g. of sample *A*, 5 g. of sample *B* and 1 g. of sample *C* (*v.s.*). The use of too little manganese dioxide makes the reaction slower than it need be, and the final yield is then below the best. Too much oxide also adversely affects the yield. Table 2 records an experiment in which solutions of a vitamin A concentrate in light petroleum (purified for spectroscopy) were prepared and 100 ml. portions left over manganese dioxide in the dark.

Table 2. *Effect of vitamin A concentration and amount of manganese dioxide on yield of retinene*

Vitamin A concentrate in petroleum (%)	Manganese dioxide		$E^{1\%}_{1\,cm.}$ 664 mμ. after 6 days
	Wt. (g.)	Sample	
0·2	5	*A*	756
0·2	5	*B*	335
0·5	5	*A*	493
1·0	5	*A*	393
1·0	25	*A*	702

These and other experiments show that for a given sample of manganese dioxide the ratio weight of manganese dioxide/weight of vitamin A is of great importance. The actual concentration of vitamin A (i.e. the amount of solvent) used was found to be much less critical.

The effects of light and of temperature. Light exerts a deleterious effect for both vitamin A and retinene are slowly destroyed in diffuse daylight. Experiments carried out in ordinary glassware kept away from direct sunlight gave very poor yields of retinene compared with similar experiments where exposure to daylight was reduced to a minimum. Other tests in which the reaction mixture was kept at 37° in the dark gave extremely poor results compared with control experiments carried out at room temperature. Warmth and light are therefore to be avoided.

Substitution of other oxidizing agents. Replacement of light petroleum by diethyl ether. Various solid oxidizing agents were tried as substitutes for manganese dioxide, but in no case could any retinene be observed. Chromium sesquioxide, chromic oxide, barium peroxide, lead peroxide, lead tetroxide and silver oxide all proved useless, and when portions of each were mixed with manganese dioxide, the yield of retinene could be accounted for by the MnO_2 moiety.

If vitamin A in light petroleum is left for long periods (e.g. 35 days) over solid oxidizing agents, other than manganese dioxide, vitamin A tends to disappear and to be replaced by a product, not retinene, exhibiting an absorption maximum near 280 mμ.

When purified diethyl ether was tried as solvent instead of light petroleum, the sole reaction product identified (and that in poor yield) was cyclized or anhydro-vitamin A.

Specificity of the oxidation. Manganese dioxide was left in the dark with light petroleum solutions of *iso*propyl alcohol, benzyl alcohol, octadecyl alcohol, cetyl alcohol and a mixture of C_{20} alcohols kindly supplied by Prof. T. P. Hilditch. In no case did chemical or spectroscopic tests for aldehydes give positive results.

Solutions of vitamin A esters in light petroleum showed little change after standing over manganese dioxide.

Ergosterol undergoes changes not yet fully elucidated, but calciferol is not readily attacked.

Purification of retinene

When the interaction between manganese dioxide and vitamin A is almost completed the mixture is filtered and the filtrate is poured on to a column of activated alumina. The best results are obtained in our experience by the use of slightly weakened alumina prepared as previously described. The chromatogram is developed by means of light petroleum:

Fraction 1. A yellow zone passes quickly down the column. The material gives a 617 mμ. band with the $SbCl_3$ reagent. It may contain a small amount of cyclized vitamin A and any vitamin A ester present in the original concentrate.

Fraction 2 travels more slowly as a broad brown zone containing most of the retinene.

Fraction 3 is a more strongly held pale-yellow zone. With the $SbCl_3$ reagent the eluate from this zone shows maxima at 617 and 664 mμ.

Fraction 4 is very firmly adsorbed. It remains as a brown zone at the top of the column and can only be removed by elution with ether or chloroform. The fraction shows no characteristic ultraviolet-absorption spectrum and the dirty red solution formed with the $SbCl_3$ reagent exhibits no well-defined maximum.

Fraction 2 is now reduced to small bulk by distilling off much of the solvent. The concentrated solution is rechromatrographed on weakened alumina. The retinene fraction is collected and examined spectroscopically. The ultraviolet absorption is maximal at 369 mμ. (in petroleum) and a value of $E^{1\%}_{1\,cm.}$ 1250 is regularly obtained. This seems to be the limit of purification attainable by simple chromatography.

It is important that the alumina should not be too active. Ordinary 'Brockman' alumina is satisfactory but the solvent percolates rather slowly, whilst the grade 0 (Peter Spence and Co.) alumina as supplied tends to produce a red artefact from retinene ($\lambda_{max.}$ 290 mμ.; $SbCl_3$ test $\lambda_{max.}$ 572→610 mμ.). By using weakened alumina the chromatography is speeded up and decomposition of retinene is eliminated.

Examination of used manganese dioxide. After filtering the reaction mixture, the manganese dioxide retains some adsorbed material which can be eluted with chloroform. The solution gives a blue colour with the Carr-Price reagent with maxima at 573, 610 and 664 mμ. If the chloroform is removed and replaced by light petroleum, ultraviolet maxima at 290 and 365 mμ. are recorded. It seems that some retinene and some of the red artefact are adsorbed on the manganese dioxide.

Crystallization of retinene

Purified light petroleum was found to be the best solvent for crystallization. The solute was a retinene fraction $E^{1\%}_{1\,cm.}$ 369 mμ. 1250 and a concentration of about 5% (w/v) was used for a preliminary cooling to −72° for 48 hr., when a small yield of feathery white crystals was obtained. This material (which was discarded) showed an absorption band at 270 mμ. in light petroleum or in chloroform, and with the $SbCl_3$ reagent a rose-coloured solution was obtained showing continuous end absorption over the whole visible spectrum range.

In addition to the above material very small amounts of a substance (also discarded) with a band of comparatively low intensity at 348 mμ. in petroleum or in chloroform were obtained. The $SbCl_3$ reagent gave a blue-coloured solution with absorption bands of approximately equal intensity at 664 and 617 mμ.

After removal of these impurities by filtering through a Buchner funnel cooled with solid carbon dioxide, the mother liquor was concentrated *in vacuo* to one-fourth its original bulk. The concentrated solution was again cooled to −72° for 24 hr. when a brown semi-solid mass was obtained. The mother liquor was decanted off and the residue dissolved in freshly distilled light petroleum to give a 5% (w/v) solution. This was kept at −72° for 10 days when a large crop of reddish brown crystals, m.p. 56·5–58°, was obtained which showed an absorption band with a single maximum at 373 mμ. in *cyclo*hexane, $E^{1\%}_{1\,cm.}$ 1354.

The material so obtained was recrystallized very carefully from 5% (w/v) solution in light petroleum, the solution being cooled slowly from 0° to −40° over a period of 7 hr. The mixture was then left at −72° for 24 hr. when large orange-red crystals were obtained in good yield. The overall yield (of crystallization) was about 20%. The crystals are large clusters predominantly needle-like in shape and melt at 61–62°. (Found: C, 84·2; H, 9·76%; mol. wt. (Rast) 236. $C_{20}H_{28}O$ requires C, 84·5; H, 9·85%; mol. wt. 284.)

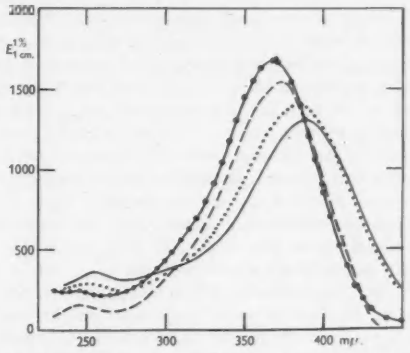


Fig. 5. Absorption spectrum of retinene$_1$ in light petroleum •—•; *cyclo*hexane — — —; ethanol · · · · · ·; and chloroform ——.

Table 3. *Absorption maxima of retinene in various solvents*

Solvent	$\lambda_{max.}$ (mμ.)	$E^{1\%}_{1\,cm.}$
Light petroleum (40–60°)	369·5	1685
*cyclo*Hexane	373	1548
Ethanol	385·5	1400
Chloroform	389	1303

The absorption spectra of crystalline retinene have been determined in various solvents. The full curves are recorded in Fig. 5 and the maximum intensity of absorption is recorded in Table 3, e.g. in *cyclo*hexane,

$E^{1\%}_{1\,cm.}$ 373 mμ., 1548; $E^{1\%}_{1\,cm.}$ 245 mμ., 159.

With the antimony trichloride reagent a blue-coloured solution was obtained, giving a single absorption band at 664 mμ., with an $E^{1\%}_{1\,cm.}$ value of 3400.

Derivatives of retinene

Retinene-2:4-dinitrophenylhydrazone. An ethanolic solution of 2:4-dinitrophenylhydrazine sulphate was added to an equivalent amount of retinene in ethanol, when an immediate darkening of the solution was observed. From this solution a good crop of crystals was obtained and the material was crystallized from ethanol and from acetone. Reddish brown needles were obtained with m.p. 207–208°. (Found: C, 66·8; H, 6·9; N, 12·2%; mol. wt. (Rast) 336. Calc. for $C_{26}H_{32}N_4O_4$: C, 67·2; H, 6·9; N, 12·1%; mol. wt. 464.) The spectrum of the derivative showed bands at 442 and 260 mμ. with $E^{1\%}_{1\,cm.}$ 1166 and 346 respectively.

Retinene semicarbazone. This derivative was prepared by adding an aqueous ethanolic solution of semicarbazide hydrochloride to an ethanolic solution of retinene. The mixture was warmed for a few minutes, and after standing overnight was extracted with ether and the semicarbazone crystallized from a mixture of ether and light petroleum. Although work has not yet been completed on this material, one sample of the derivative, which must have been of high purity, had m.p. 161–164° and an absorption band at 385 mμ. in chloroform with $E^{1\%}_{1\,cm.}$ 1742. On standing in solution at 0° for some weeks, the intensity of the 385 mμ. band had decreased with the production of another band of equal intensity at 365 mμ. These data are at variance with those reported by van Dorp & Arens (1947) who state that this derivative has m.p. 207–209° (with decomposition), an absorption spectrum $\lambda_{max.}$ 375 mμ., log $E_{max.}$ 4·87, and reacts in dilute solutions with chloroformic $SbCl_3$ to give a purple-red colour and in stronger solutions a blue-violet colour. Comparison with our data is difficult for they do not state the solvent used to determine the ultraviolet spectrum and the colour-test statements are too qualitative.

Retinene hydrazone derivatives. Two hydrazone derivatives of retinene have been obtained by reaction of a solution of hydrazine hydrate with retinene followed by fractional crystallization. Work has not yet been completed but the following details have been obtained.

Derivative A, m.p. 108–109°. Single broad band absorption maximum in ethanolic solution.

$\lambda_{max.}$ 355 mμ.; $E^{1\%}_{1\,cm.}$ 280.

Derivative B, m.p. 135–145°. Two absorption bands in ethanolic solution.

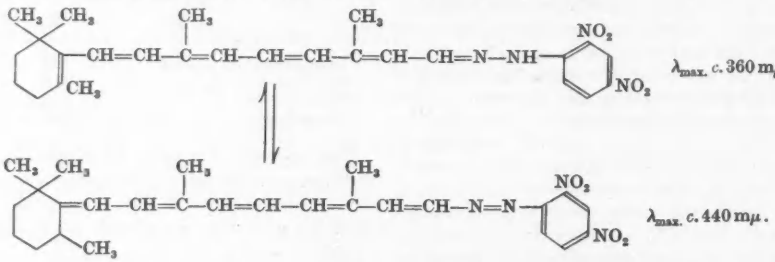
$\lambda_{max.}$ 460 mμ.; $E^{1\%}_{1\,cm.}$ 1502
298 mμ.; 322.

With pure retinene, a semicarbazone, m.p. 193–195° and $E^{1\%}_{1\,cm.}$ 385 mμ. 2062, and a 'hydrazone', m.p. 177°, $\lambda\lambda_{max.}$ 462 and 297 mμ., $E^{1\%}_{1\,cm.}$ 1930 and 334 respectively, have since been obtained. The semicarbazone analyzed correctly. Found: C, 73·8; H, 9·1; N, 12·3. Calc. for $C_{21}H_{31}N_3O$: C, 73·0; H, 9·1; N, 12·5%. The '*hydrazone*' analyzed correctly for an azine. Found: C, 85·1; H, 10·0; N, 4·74; mol. wt. 500. $C_{40}H_{56}N_2$ requires C, 85·1; H, 9·9; N, 4·9%; mol. wt. 564.

DISCUSSION

The preparation described above forms a convenient and economical method of preparing retinene. The material so formed shows the same spectral characteristics as that isolated from retinas by Wald (1935–6), and differs from the materials prepared by Morton & Goodwin (1944) and by Hunter & Hawkins (1944) only in its state of purity. Hunter & Hawkins obtained a material showing absorption bands at 368 and 350 mμ. but it is now apparent that the 350 mμ. band is not characteristic of retinene but is due to an impurity, probably an artefact. This artefact has been isolated in an impure state and in small yield and may possibly be a polymerization product of retinene.

The preparation of the retinene crystals and of its derivatives gives proof to the suggestion of Morton (1944) that retinene is the aldehyde of vitamin A. Some points of doubt, however, still remain to be investigated completely, e.g. the absorption spectrum of retinene-2:4-dinitrophenylhydrazone is atypical, though it is possible that the following equilibrium may exist:



The low molecular weights obtained for this compound and for retinene were obtained by Rast's method, and it is reasonable to believe that these results are due to the rather severe treatment required by this method. A further point of doubt concerns the two hydrazone derivatives, although the spectral evidence indicates that *derivative A* is the normal hydrazone type and *derivative B* is, in all probability, an azine compound of the type

$$R\text{—CH=CH—CH=N—N=CH—CH=CH—}R.$$

The semicarbazone derivative prepared seems normal, and the production of the 365 mμ. band on standing in solution can be explained by assuming the existence of the following dynamic equilibrium (see p. 523).

The conversion of vitamin A alcohol to retinene depends upon the surface catalysis of manganese

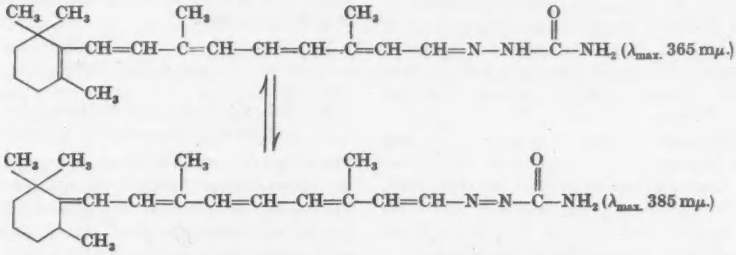
dioxide. In all probability the process is triphasic, the three stages comprising:

(1) The removal of vitamin A alcohol from the light petroleum phase by adsorption on manganese dioxide.

(2) The conversion of the CH_2OH group to the CHO group.

(3) The passage of retinene into the petroleum phase.

The oxidation, as far as our investigations go, is specific, for replacement of either of the reactants



(i.e. vitamin A or manganese dioxide) by a similar substance prevents any comparable reaction. The oxidation process is dependent on two facts, viz. that vitamin A is more strongly adsorbed on manganese dioxide than is retinene, and that the solid adsorbent is capable of converting the primary-alcoholic group to the aldehyde group without attacking other parts of the molecule. Apart from the importance of the reaction from a purely preparative point of view, it may well be that it is of deeper significance. The mechanism connecting vitamin A with retinene in the cycle of scotopic vision is a matter of some doubt, but it is possible that one stage of the retinal process is a surface oxidation analogous to the one described above.

SUMMARY

1. A simple method of preparing retinene (vitamin A aldehyde) from vitamin A in excellent yield is described.

2. Retinene has been obtained crystalline and fully characterized.

3. The preparation of derivatives of retinene with 2:4-dinitrophenylhydrazine, hydrazine and semicarbazide is described.

4. Absorption spectra of retinene and its derivatives are given.

We are indebted to the Medical Research Council and the Ministry of Food for financial assistance.

REFERENCES

Dorp, D. A. van & Arens, J. F. (1947). *Nature, Lond.*, **160**, 189.

Edisbury, J. R., Morton, R. A. & Simpkins, G. W. (1937). *Nature, Lond.*, **140**, 234.

Granit, R. (1947). *Sensory Mechanisms of the Retina.* Oxford: The University Press.

Hunter, R. F. & Hawkins, E. G. E. (1944). *J. Chem. Soc.* p. 411.

Lederer, E. & Rosanova, V. (1937). *Biochimia*, **2**, 293.

Morton, R. A. (1944). *Nature, Lond.*, **153**, 69.

Morton, R. A. & Goodwin, T. W. (1944). *Nature, Lond.*, **153**, 405.

Morton, R. A. & Stubbs, A. L. (1946). *Analyst*, **71**, 348.

Wald, G. (1935–6). *J. gen. Physiol.* **19**, 351.

Wald, G. (1937). *Nature, Lond.*, **140**, 545.

Wald, G. (1938–9*a*). *J. gen. Physiol.* **22**, 391.

Wald, G. (1938–9*b*). *J. gen. Physiol.* **22**, 775.

Proteolytic Activity of Preparations of Crystallized Ribonuclease

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The first preparations of ribonuclease were made by methods involving heating (Jones, 1920; Dubos & Thompson, 1938), but since the adoption of the Kunitz (1940) method for the isolation of the enzyme in crystalline form, this step has been omitted. It appears to have been assumed that crystallinity presupposes homogeneity, and crystallized preparations of the enzyme, which have never been exposed to heat, have been used by many workers under conditions in which the presence of other enzymes, and of protease in particular, renders interpretation of results uncertain. It has been established with many proteins that crystallinity is no guarantee of purity (Pirie, 1940) and this seems to apply to ribonuclease. Cohen (1945), when studying the effect of proteolytic enzymes on a

thymus nucleohistone, and Stowell & Zorzoli (1947), using ribonuclease in cytochemical work, detected proteolytic activity in the ribonuclease crystallized by Kunitz's method. Cohen stated that it is impossible to decide whether ribonuclease itself possesses proteolytic activity or whether the crystalline preparations were contaminated with protease.

In attempts to determine the action of ribonuclease on plant viruses, many different crystalline preparations of the enzyme have been prepared. All these have been found to possess proteolytic activity, but the ratio of ribonuclease to proteolytic activity has varied in different preparations, although all of them have otherwise seemed similar. Hence it seems more likely that the preparations are mixtures of enzymes rather than that ribonuclease itself has proteolytic activity.

The simultaneous occurrence of proteolysis and ribonuclease activity could, of course, invalidate many of the results obtained in cytochemical work, or with such substrates as potato virus X, which are affected by proteolytic enzymes. For the unequivocal interpretation of results with ribonuclease, it is essential that proteolytic activity should be excluded, and this paper describes methods whereby this can be achieved. It also describes the effect of pepsin and chymotrypsin on ribonuclease, as well as the effect of the protease present in its crystallized preparations.

MATERIAL AND METHODS

Enzymes. Ribonuclease was prepared from beef pancreas and crystallized from $(NH_4)_2SO_4$ solutions by the method of Kunitz (1940). Small amounts of the enzyme could also be obtained in crystalline form by complete saturation with $(NH_4)_2SO_4$ of the filtrates which are obtained after precipitation of most of the enzyme at 80% saturation. The enzyme was usually crystallized twice, dissolved in, and dialyzed against, distilled water, and then dried in flat dishes in a desiccator at 37°. It was stored as a dry powder, which dissolved readily in water or in buffer solutions.

Crystalline chymotrypsin was prepared from beef pancreas by the method described by Northrop (1939). It was crystallized eight times as chymotrypsinogen, then converted into chymotrypsin and twice crystallized. The crystals were dissolved and dialyzed against distilled water, dried and stored as a powder.

The pepsin used was a commercial preparation obtained from the British Drug Houses Ltd.

The following buffer solutions were used: pH 2·0–4·9, 0·1 M-Sorensen citrate-HCl; pH 4·9–6·6, 0·1 M-Sorensen citrate-NaOH; pH 6·8–8·0, 0·2 M-boric acid-borax.

Determination of enzyme activities. Ribonuclease activity was measured by estimating the amounts of yeast nucleic acid rendered non-precipitable by acetic acid according to the method previously described (Kleczkowski, 1946). A commercial preparation of yeast nucleic acid (British Drug Houses Ltd.) was used for the purpose; about 40% of the nucleic acid was precipitated when 10 vol. of glacial acetic acid were added to 1 vol. of a neutral solution.

Proteolytic activity was measured by estimating the amount of casein rendered soluble in 1% trichloroacetic acid. One vol. of the solution under test was mixed with 2 vol. of a 1% solution of casein and the mixtures incubated at 37°. Trichloroacetic acid (10% w/v) was then added to bring the concentration to 1%. The precipitates were centrifuged down and the nitrogen in the supernatant fluids was estimated. If the mixtures were incubated at pH values other than 6–7, they were neutralized before adding trichloroacetic acid. Enzyme concentrations and times of incubation varied in different experiments.

Other determinations. Phosphorus and nitrogen determinations were made by the methods previously described (Kleczkowski, 1946).

EXPERIMENTAL

The effect of pH *on the ribonuclease and proteolytic activities of preparations of ribonuclease*

The identity of the protease contaminating crystalline preparations of ribonuclease is uncertain, but its pH range of activity suggests similarities with trypsin and chymotrypsin. When tested at pH 7·2 with casein as substrate, 1 mg. of an average preparation of ribonuclease usually has a proteolytic effect equivalent to that of 0·02 mg. of crystalline chymotrypsin. Thus if the contaminating protease were chymotrypsin, it would be present in ribonuclease to the extent of about 2%, and less if it were trypsin.

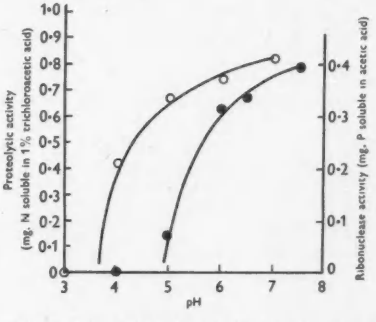

Fig. 1. The effect of pH on proteolytic and ribonuclease activities of a ribonuclease preparation. Proteolytic activity: 1 ml. of 0·1% ribonuclease in water was mixed with 2 ml. of 1% casein solution in a buffer of required pH, and the mixtures were incubated for 24 hr. at 37°. Ribonuclease activity: 0·5 ml. of 0·001% ribonuclease solution in a buffer of required pH was mixed with 0·5 ml. of 3% solution of yeast nucleic acid in the same buffer, and the mixtures were incubated for 30 min. at 50°. ○—○, ribonuclease activity; ●—●, proteolytic activity.

The effect of pH on the proteolytic and ribonuclease activities of a representative ribonuclease preparation is shown in Fig. 1. No ribonuclease

activity could be detected at pH 3, but it became appreciable between pH 3 and 4, and increased rapidly with increasing pH values. There was no detectable proteolytic activity at pH 4; it appeared as the pH approached 5, and increased rapidly above pH 5. It is thus possible to obtain a relatively slight ribonuclease activity in the absence of proteolytic activity by incubating in the pH range 4·0–4·5.

The effect of heat on the proteolytic and ribonuclease activities of preparations of ribonuclease

The earlier work on the preparation of ribonuclease paid little attention to the conditions of heating. Jones (1920) boiled aqueous extracts of pig's pancreas, and Dubos & Thompson (1938), after extracting commercial pancreatin with 50% acetone, precipitated the enzyme by adding more acetone, dissolved the precipitated enzyme in water and heated it in boiling water. No control of the pH seems to have been made during heating, and no tests are described for the effect of heating on proteolytic activity. After Kunitz (1940) had crystallized the enzyme, he estimated its heat stability over a range of pH values, but did not test his preparations for enzymic activities other than ribonuclease activity.

The effects of heat on the proteolytic and ribonuclease activities were investigated as follows. Tubes of 15 mm. diameter, containing 2 ml. of 0·1% ribonuclease in buffers at different pH values, were immersed in boiling water for 5 min. To prevent evaporation, the tubes were closed with rubber stoppers through which capillary tubes were inserted. The temperature of the heated fluids did not reach 100°, but increased in the following way after immersion in the water bath: after 1 min., 90°; 2 min., 96·5°; 3 min., 98·5°; 4 min., 99·0°; 5 min., 99·25°. After 5 min., the tubes were rapidly cooled, the pH in all was adjusted to 7 and the volume to 4 ml.; the proteolytic and ribonuclease activities of the solutions were then determined.

It will be seen from Fig. 2 that at pH values between 2 and 3 proteolytic activity was less affected by heat than was ribonuclease activity; as the pH increased the susceptibility of proteolytic activity to heat increased progressively, whereas that of ribonuclease remained apparently unchanged until the pH reached about 6, after which it increased rapidly. After heating between pH 7·0 and 8·0 proteolytic activity could not be detected, whereas ribonuclease activity, though decreased, was still present; this also disappeared as a result of heating at pH 8·0.

Somewhat different results may be obtained by changing the conditions of heating. For example, by varying the volumes of heated ribonuclease solutions or the diameter of tubes in which they are heated, the rate of temperature increase is changed, and as a result the drop in ribonuclease activity is different. The difference is particularly pronounced in the pH range where the rate of inactivation of the enzyme is relatively high, i.e. at pH values higher than 6. The dependence of heat inactivation of ribonuclease on the pH, as shown in Fig. 2, is on the whole in agreement with the results obtained by Kunitz (1940). The differences, notably the much greater inactivation at pH values higher than 6 found by Kunitz, are almost certainly due to different conditions of heating.

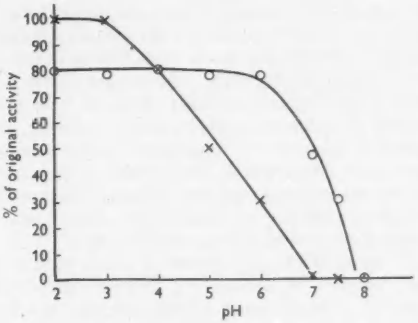

Fig. 2. The effect of pH on heat stability of proteolytic and ribonuclease activities of a preparation of ribonuclease. O—O, ribonuclease activity; ×—×, proteolytic activity.

These results suggested that suitable conditions of heating might be found that would destroy proteolytic activity while leaving satisfactorily active preparations of ribonuclease. The effect of heating was therefore studied in greater detail.

When tubes of 15 mm. diameter, containing 2 ml. of 0·1% ribonuclease solution in 0·2 M-boric acid-borax buffer at pH 7·5, were immersed for 10 min. in a water bath at 80°, ribonuclease activity was reduced by only 20%, whereas the proteolytic activity was so reduced that it was not possible to estimate it accurately by the method described above. When 1 ml. of a 0·1% solution of heated ribonuclease was incubated with 2 ml. of 1% casein solution at pH 7·5 for 24 hr. at 37° it failed to render any measurable amount of the casein non-precipitable by 1% trichloroacetic acid. Some residual proteolytic activity remained, however, and it could be measured by using the more accurate method of Pope & Stevens (1939) as modified by Tracey (1948). This method is based on the decomposition of a suspension of copper phosphate by the amino groups liberated from the protein substrate (gelatin). The estimations were kindly made by Mr M. V. Tracey.

After heating in a water bath at 80° under the conditions given above, the proteolytic activity of ribonuclease solutions was reduced to about one-fiftieth of that of the unheated preparation. After heating for 5 min. in boiling water in otherwise similar conditions no proteolytic activity could be

detected even by the more sensitive test, whereas about 30% of the original ribonuclease activity still remained. The heating should not be prolonged beyond 5 min., as ribonuclease activity is then further reduced; after 10 min. only 5% of the original ribonuclease activity remains, and after 15 min. none can be detected.

No significant differences were found between the heat stabilities of crystalline and uncrystallized preparations of the enzyme, although the latter contained about ten times more proteolytic activity per unit weight. Preparations made simply by precipitation with 80% saturated ammonium sulphate, instead of by crystallization, when heated at pH 7·5 for 5 min. in boiling water, lost all detectable proteolytic activity but retained about 30% of their original ribonuclease activity. During heating, considerable amounts of coagulum usually separate from these preparations. Crystalline preparations, on the other hand, usually remain almost clear, though sometimes an opalescence, which can be removed by centrifugation, may develop.

In the light of these results it seems that if the heat method is applied to destroy proteolytic activity, crystallization of the enzyme is of no advantage for many kinds of purely qualitative work, particularly as the yields of amorphous enzyme by precipitation with ammonium sulphate (80% saturated) are higher than those obtained by crystallization.

Action of unheated and heated ribonuclease on yeast nucleic acid

Solutions of unheated and of heated ribonuclease in concentrations giving equivalent activity as indicated by their ability to render yeast nucleic acid non-precipitable by acetic acid, were also compared for their ability to produce dialyzable products from yeast nucleic acid.

Three 5 ml. samples of a 3% aqueous solution of commercial yeast nucleic acid at pH 6·0 were mixed respectively with 5 ml. of 0·001% solution of unheated ribonuclease, with 5 ml. of 0·0033% solution of heated ribonuclease, both in 0·2M-boric acid-borax buffer at pH 6·8, and with 5 ml. of the buffer. (The ribonuclease was heated as a 0·1% solution in 0·2M-boric acid-borax buffer at pH 7·5, and was diluted 1 : 30 in the pH 6·8 buffer.) After the mixtures had been incubated for 30 min. at 50°, 1 ml. samples were taken from each and mixed with 10 ml. of glacial acetic acid. The precipitates were spun down, washed in glacial acetic acid and their P contents estimated. These were 0·200, 0·195 and 0·800 mg., respectively, in the precipitates from the mixtures with unheated ribonuclease, heated ribonuclease and with the buffer alone.

The remainder of the digests was divided into two portions of 4·5 ml. each. These were placed in separate test tubes, the openings of which were then closed with cellophan membranes held in position with rubber bands. Each tube was inverted and the membrane immersed in a wider tube containing 5 ml. water. The tubes were left for 6 hr. at room temperature and the P contents of the outside and inside fluids were estimated.

Table 1. *Formation of dialyzable products from yeast nucleic acid as a result of incubation with unheated and with heated solutions of ribonuclease*

Addition to yeast nucleic acid	Test no.	Phosphorus content (mg. P) Outside fluid	Inside fluid
Unheated ribonuclease	1	1·23	3·75
	2	1·20	3·77
Heated ribonuclease	3	1·25	3·74
	4	1·22	3·81
Buffer (control)	5	0·44	4·51
	6	0·42	4·55

From the results shown in Table 1 it will be seen that some of the yeast nucleic acid incubated with the buffer alone was dialyzable, but the amount of dialyzable material was increased to approximately the same extent by incubation both with the unheated and heated ribonuclease. Kunitz (1940) found that his crystalline preparations of ribonuclease caused the formation of dialyzable products from yeast nucleic acid, but in an earlier work Schmidt & Levene (1938), using ribonuclease prepared from commercial pancreatin by the method of Dubos & Thompson (1938), could not detect their formation. Tipson (1945) suggested that this discrepancy might be due to the fact that Schmidt & Levene (1938) used heated enzyme preparations, whereas Kunitz used unheated enzyme. This possibility would seem to be excluded by the results described above. If no such products were formed by the preparations used by Schmidt & Levene, it seems probable that their ribonuclease was not identical with that which is obtained from fresh beef pancreas by the method of Kunitz. This cannot be assumed with certainty, however, for an alternative explanation, suggested by Loring & Carpenter (1943), is that Schmidt & Levene (1938) failed to detect any dialyzable products because these were retained by the proteins present in their enzyme preparations.

The effect of proteolytic enzymes on ribonuclease

For these experiments, the ribonuclease solutions were dissolved either in 0·1M-citrate-HCl buffer at pH 2·5 or in 0·2M-boric acid-borax buffer at pH 7·5, and the solutions of pepsin and of crystalline chymotrypsin, neither of which showed any detectable ribonuclease activity, were dissolved in water. The mixtures were incubated at 37°. Samples were taken at stated intervals, diluted 1 : 25 and 1 : 50 in 0·2M-boric acid-borax buffer at pH 6·8, mixed with equal volumes of 3% solution of yeast nucleic acid in water at pH 6·0, and incubated for 30 min. at 50°. The amounts

of P in the material precipitable with acetic acid were estimated, and percentages of the ribonuclease activities were determined by comparison with control enzyme solutions, as previously described (Kleczkowski, 1946).

Table 2 summarizes the results of experiments in which the susceptibility of heated and unheated ribonuclease to proteolytic enzymes was thus tested. Both pepsin and chymotrypsin destroyed ribonuclease, the former being the more effective. No ribonuclease activity could be detected after 24 hr. incubation with pepsin, whereas one-third of the ribonuclease activity was still present after 48 hr. incubation with chymotrypsin.

It can also be seen that at pH 7·5, but not at pH 2·5, ribonuclease was hydrolyzed by the protease present in its crystalline preparation; this is shown by the fact that incubation at pH 7·5 decreased ribonuclease activity of the unheated preparation (test no. 4), but not that of the heated one (test no. 6) in which proteolytic activity had been destroyed. The ribonuclease activity of material used in tests nos. 5 and 6 corresponded to about 70 % of that used in tests nos. 1–4. When control mixtures, similar to those shown in Table 2, were stored at 2° instead of being incubated at 37°, no detectable drop in ribonuclease activity occurred in any of them.

Dubos & Thompson (1938), using preparations of ribonuclease prepared by their method from commercial pancreatin, found that ribonuclease was hydrolyzed by pepsin, but remained unaffected by trypsin or chymotrypsin. Whether their enzyme was really resistant to trypsin and chymotrypsin, or whether their failure to detect any effect was due to experimental conditions, is uncertain. If the former is correct, then it seems that their ribonuclease differs from our preparations.

Table 2. *The effect of pepsin, chymotrypsin and of ribonuclease-protease on ribonuclease*

	Contents of mixtures (ml.)						Ribonuclease activity (as % of the original) after:		
Test no.	Unheated ribonuclease (0·05 %)	Heated ribonuclease (0·1 %)	Pepsin (0·05 %)	Chymotrypsin (0·05 %)	Buffer	pH	0 hr.	24 hr.	48 hr.
1	1	—	1	—	—	2·5	100	0	—
2	1	—	—	—	1	2·5	100	100	—
3	1	—	—	1	—	7·5	100	60	30
4	1	—	—	—	1	7·5	100	85	70
5	—	1	—	1	—	7·5	100	60	30
6	—	1	—	—	1	7·5	100	100	100

SUMMARY

1. Crystalline preparations of ribonuclease invariably possessed proteolytic activity, which varied in amount in different preparations. The pH zones of proteolytic and ribonuclease activities are somewhat different, and between pH 4·0 and 4·5 only the ribonuclease activity, though far from its optimum, is demonstrable.

2. By heating at pH 7·0–7·5 under specified conditions, the proteolytic activity can be greatly reduced or destroyed, leaving between 30–80 % of the original ribonuclease activity.

3. Ribonuclease is susceptible to both pepsin and chymotrypsin, but it is hydrolyzed much more rapidly by pepsin. The proteolytic enzyme present in crystalline preparations of ribonuclease also hydrolyzes ribonuclease.

REFERENCES

Cohen, S. S. (1945). *J. biol. Chem.* **158**, 255.
Dubos, R. J. & Thompson, R. H. S. (1938). *J. biol. Chem.* **124**, 501.
Jones, W. (1920). *Amer. J. Physiol.* **52**, 203.
Kleczkowski, A. (1946). *Biochem. J.* **40**, 677.
Kunitz, M. (1940). *J. gen. Physiol.* **24**, 15.
Loring, H. S. & Carpenter, F. H. (1943). *J. biol. Chem.* **150**, 381.
Northrop, J. H. (1939). *Crystalline Enzymes.* New York: Columbia University Press.
Pirie, N. W. (1940). *Biol. Rev.* **15**, 377.
Pope, C. G. & Stevens, M. F. (1939). *Biochem. J.* **33**, 1070.
Schmidt, G. & Levene, P. A. (1938). *J. biol. Chem.* **126**, 423.
Stowell, R. E. & Zorzoli, A. (1947). *Stain Tech.* **22**, 51.
Tipson, R. S. (1945). *Adv. Carbohydrate Chem.* **1**, 193.
Tracey, M. V. (1948). *Biochem. J.* **43**. (In the Press.)

The Effects of Various Organic Materials on Soil Nitrification

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In his early work on the isolation of nitrifying organisms from various soils Winogradsky (1891) found that the organisms could not be cultured on gelatin. Winogradsky & Omeliansky (1899, 1902) showed that various organic materials (glucose, gelatin, asparagine, peptone, etc.) were actually toxic to the nitrifiers in culture. The more recent work on nitrification does not entirely confirm these findings. Kingma Boltjes (1935) observed nitrification in the presence of glucose, and succeeded in showing that the inhibitory effect of peptone was due to the presence of free amino-acids.

Hes (1937) in studying *Nitrosomonas* not only cultured his organisms on agar, but found that the addition of an egg preparation actually stimulated their growth, thus confirming the results of Fred & Davenport (1921) and of Kingma Boltjes (1935). Bömecke (1939), on the basis of the results of Warburg respiration experiments, even claimed to have shown a small but definite heterotrophic dissimilation in nitrifying cultures. Nevertheless, the vague concept that 'organic matter is toxic to nitrification', which arose from the work of Winogradsky & Omeliansky (1899), is still current in the literature on nitrification, and within the last 10 years Pandalai (1946) and Desai & Fazal-ud-Din (1937) have been led to put forward theories of symbiosis to account for the fact that nitrification proceeds in the presence of organic matter. But the situation remains obscure, and the present work was begun in order to determine whether there is in fact any need for a theory of symbiosis to account for soil nitrification and to discover whether the various organic materials reported as being toxic in culture have any direct effect on the nitrification process in soil.

METHODS

The percolation technique previously described (Lees & Quastel, 1946*a*) was used throughout, but the percolation apparatus was the simplified and improved form described by Lees (1947). Measurements of the time taken for ammonium ions, freshly added to the percolate, to reach an equilibrium with the soil exchange complexes have shown that equilibrium is reached within 3 hr. Because this equilibrium is reached so quickly, it has been possible to conduct replicable nitrification experiments of only 5 hr. duration.

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The garden soil containing 0·4% organic N used in previous experiments (Lees & Quastel, 1946 *a*, *b*, *c*) has been used in all the present work. It is herein described as the allotment soil. Except where otherwise stated experiments were carried out on 10 g. of this soil (air dry and sieved to 4·0–1·0 mm. mesh) and 100 ml. percolate.

Determinations of the various forms of inorganic N were made by methods similar to those described by Lees & Quastel (1946*a*).

Of organic materials 10 ml. at 1% concentration were added to 90 ml. percolate. The gelatin and peptone solutions were dialyzed against 100 vol. distilled water for 24 hr. before use, in order to minimize the possible toxic effect of free amino-acids noted by Kingma Boltjes (1935). The 'humus' was prepared at pH 7 from a Littleport fen soil by a pyrophosphate extraction (Bremner & Lees, in course of preparation).

Calculation of results. The values of 'μg. NO_3-N formed/g. soil' given in the tables were calculated from percolate analyses by methods similar to those already described (Lees & Quastel, 1946*a*).

Because the experimental period was necessarily short, the measured formation of NO_2-N or NO_3-N in the percolates was small (only some 3–4 μg. N/ml.). The reagent blank on the phenoldisulphonic acid estimation was equivalent to about 1·0 μg. NO_3-N/ml. percolate or about 25% of the actually estimated amount of nitrate. This reagent blank seems unavoidable; it appears to be due mainly to the H_2O_2 used for oxidation of nitrite in the sample. Fortunately a reagent blank introduces only a small error into comparative colorimetric readings if all the 'test' readings are close to one another and are read against the same standard, and even this small error has been eliminated by the use of the following easily derived equation in the calculation of the results:

$$U = \frac{S\,(T - B)}{(S - B)},$$

where S = nominal concentration of standard reference solution; T = colorimetrically observed concentration of test solution X; B = colorimetrically observed concentration of blank solution; and U = true concentration of test solution X.

Experimental procedure. The design of a valid experimental procedure to test the effect of organic materials on the nitrification of ammonium ions by soil is not simple. The nitrifying organisms have a considerable lag phase in their proliferation curve; nitrification of ammonium ions percolated through the allotment soil is not appreciable during the first 3 days of the experiment and is not well established before the 6th (Lees & Quastel, 1946*a*). On the other hand heterotrophic proliferation in response to the addition of a carbon source to the percolate is likely to result in a relatively enormous immobilization of mineral N during the first 4 days (Fig. 1). This figure shows only the immobilization of NO_3-N in the presence of various organic materials,

but similar curves are obtained if $(NH_4)_2SO_4$ is substituted for $NaNO_3$ in the percolate. The gradual reappearance of NO_3-N in the percolates after 4 days indicated that all the carbon of the added organic material had been consumed

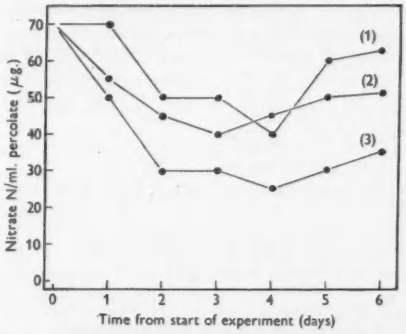

Fig. 1. The immobilization of nitrate N by organic materials percolated through allotment soil. Allotment soil (10 g.) percolated with 100 ml. 10^{-2}M-$NaNO_3$ containing: curve 1, sucrose; curve 2, glucose; curve 3, glycerol; all at 0·4 mg. C/ml.

and that lysis of a moribund heterotrophic population was taking place. The difficulty of following nitrification (i.e. the formation of nitrite and subsequently nitrate, from ammonium ions) under these conditions was largely overcome by the use of the 'stimulated soil' technique, already described by Lees & Quastel (1946*c*), in which soils are stimulated to active nitrification before the experiment proper. Such soils begin to nitrify fresh $(NH_4)_2SO_4$ immediately it is percolated; there is no lag phase in the process. It has been found that nitrification can proceed to some measurable extent in these 'stimulated soils' before breakdown of any organic material added to the percolate causes serious immobilization of either ammonium or nitrate ions.

The procedure used to test the effect of organic materials on soil nitrification was therefore as follows. Allotment soil in 10 g. lots was stimulated to active nitrification by percolation with 100 ml. of $2{\cdot}5 \times 10^{-3}$M-$(NH_4)_2SO_4$ for 12 days at 18–21°. The samples were then washed 3 times with 50 ml. lots of distilled water and finally washed by percolation with 100 ml. distilled water for 24 hr.; this percolate and the previous washings were discarded. All detectable nitrite and nitrate were thus removed from the soils, which were then percolated either with 100 ml. of $2{\cdot}5 \times 10^{-3}$M-$(NH_4)_2SO_4$ alone, or with 100 ml. of $2{\cdot}5 \times 10^{-3}$M-$(NH_4)_2SO_4$ + 0·1% of the organic material under test. Nitrite and nitrate formation by the soils in the presence and absence of organic material were determined by percolate analyses.

RESULTS

The first experiments were of 24 hr. duration. The formation of nitrite N and nitrate N during this period by soils percolated with ammonium sulphate alone and with ammonium sulphate plus glucose (or starch) are given in Table 1. Similar soils percolated with sodium nitrate, and with sodium nitrate plus 0·1% glucose (or starch) served as controls. The virtual absence of any nitrite formation by the control soils demonstrated that the appreciable quantities of nitrite formed by the test soils percolated with ammonium sulphate plus glucose (or starch) could not have arisen by reduction of traces of nitrate left in the soil. The nitrite formation observed in the presence of glucose and starch must have been from ammonium ions. This shows that in the allotment soil the oxidation of ammonium ions to nitrite ions, i.e. the first stage of nitrification, can proceed in the presence of 0·1% glucose or 0·1% starch. Nevertheless, before the 24 hr. were complete the glucose, but not the starch, percolates were showing a cloudiness, indicative of some heterotrophic proliferation that might have accounted for the obviously diminished nitrate-N formation by the glucose percolated soils. The next experiments, performed on a wider range of organic materials, were therefore of only 5 hr. duration (Table 2).

The results in Table 2 are complicated by breakdown of gelatin and peptone, but they agreed with those of Table 1 in showing that the organic materials tested had little effect on soil nitrification. This was checked by an experiment in which only 50 ml. of percolate were used (Table 3), and in which the concentration of nitrate N was reduced from 70 μg. NO_3-N/ml. to 10 μg. NO_3-N/ml. The results obtained agreed satisfactorily with those of Table 2, not only qualitatively but quantitatively. The quantitative agreement indicates that the estimation methods used for nitrite N and nitrate N were reliable, even though the total quantity of nitrogen estimated was only a few μg./ml.

Table 1. *The formation of nitrite* N *and nitrate* N *by* 10 *g. stimulated allotment soil in the presence of glucose and starch*

(Duration of experiment, 24 hr.)

Soil percolated with 100 ml. of	Nitrite N or nitrate N formed/g. soil (μg.)		
	Nitrite N	Nitrate N	Nitrite N + nitrate N
$2{\cdot}5 \times 10^{-3}$M-$(NH_4)_2SO_4$	20	220	240
$2{\cdot}5 \times 10^{-3}$M-$(NH_4)_2SO_4$ with 0·1% glucose	15	130	145
$2{\cdot}5 \times 10^{-3}$M-$(NH_4)_2SO_4$ with 0·1% starch	12	200	212
5×10^{-3}M-$NaNO_3$	0·1	—	—
5×10^{-3}M-$NaNO_3$ with 0·1% glucose	0·1	—	—
5×10^{-3}M-$NaNO_3$ with 0·1% starch	0·1	—	—

Table 2. *The formation of nitrite* N *and nitrate* N *by* 10 *g. stimulated allotment soil in the presence of various organic materials*

(Duration of experiment, 5 hr.)

Soil percolated with 100 ml. of	Nitrite N or nitrate N formed/g. soil (μg.)		
	Nitrite N	Nitrate N	Nitrite N + nitrate N
$2{\cdot}5 \times 10^{-3}$ M-$(NH_4)_2SO_4$	11, 16	31, 20	42, 36
$2{\cdot}5 \times 10^{-3}$ M-$(NH_4)_2SO_4$ and 0·1 % glucose	7, 15	32, 20	39, 35
$2{\cdot}5 \times 10^{-3}$ M-$(NH_4)_2SO_4$ and 0·1 % agar	7, 13	31, 19	38, 32
$2{\cdot}5 \times 10^{-3}$ M-$(NH_4)_2SO_4$ and 0·1 % gelatin	17	18	35
$2{\cdot}5 \times 10^{-3}$ M-$(NH_4)_2SO_4$ and 0·1 % peptone	31, 13	0, 22	31, 35
$2{\cdot}5 \times 10^{-3}$ M-$(NH_4)_2SO_4$ and 0·1 % humus	16	14	30
5×10^{-3} M-$NaNO_3$ and 0·1 % glucose	0·1, 0·1	—	—
5×10^{-3} M-$NaNO_3$ and 0·1 % agar	0·1, 0·1	—	—
5×10^{-3} M-$NaNO_3$ and 0·1 % gelatin	5	—	—
5×10^{-3} M-$NaNO_3$ and 0·1 % peptone	22, 5	—	—
5×10^{-3} M-$NaNO_3$ and 0·1 % humus	0·1	—	—
Water alone	0·1	*c.* 1	*c.* 1

Table 3. *The formation of nitrite* N *and nitrate* N *by* 10 *g. stimulated allotment soil in the presence of various organic materials*

(Duration of experiment, 5 hr.)

Soil percolated with 50 ml. of	Nitrite N or nitrate N formed/g. soil (μg.)		
	Nitrite N	Nitrate N	Nitrite N + nitrate N
$2{\cdot}5 \times 10^{-3}$ M-$(NH_4)_2SO_4$	22	14	36
$2{\cdot}5 \times 10^{-3}$ M-$(NH_4)_2SO_4$ and 0·1 % glucose	22	15	37
$2{\cdot}5 \times 10^{-3}$ M-$(NH_4)_2SO_4$ and 0·1 % agar	22	8	30
$2{\cdot}5 \times 10^{-3}$ M-$(NH_4)_2SO_4$ and 0·1 % gelatin	18	12	30
$2{\cdot}5 \times 10^{-3}$ M-$(NH_4)_2SO_4$ and 0·1 % peptone	26	4	30
$2{\cdot}5 \times 10^{-3}$ M-$(NH_4)_2SO_4$ and 0·1 % humus	20	10	30
5×10^{-4} M-$NaNO_3$	0	0	0
5×10^{-4} M-$NaNO_3$ and 0·1 % glucose	0	0	0
5×10^{-4} M-$NaNO_3$ and 0·1 % agar	0	0	0
5×10^{-4} M-$NaNO_3$ and 0·1 % gelatin	8	3	11
5×10^{-4} M-$NaNO_3$ and 0·1 % peptone	11	3	14
5×10^{-4} M-$NaNO_3$ and 0·1 % humus	0	0	0

DISCUSSION

The results reported show that if the organic materials that are supposed to inhibit nitrification in culture do so in soil, the inhibitions by 0·1 % of the materials tested are small. Moreover, if the lack of inhibition is due to a symbiosis between heterotrophs and nitrifiers, the symbiosis is either established with startling rapidity, or exists preformed in a soil not previously treated with organic material under test. Although results show reasonably clearly that 0·1 % gelatin has little effect on nitrification in soil, this does not mean that gelatin could have no effect on nitrification in culture at a similar concentration. Gelatins always contain 5–50 p.p.m. of lead, zinc and copper, and the actual balance of at least the last two of these trace elements may profoundly affect the speed of nitrification in culture (Meiklejohn & Lees, 1948). If, however, the reported inhibitions of culture nitrification were in fact due to trace-element disturbances, there is no possibility of demonstrating the same effect in a normal soil because in it the trace-element balance is strongly buffered by soil-complex reserves of these elements.

The present work seems to contradict much published work on the inhibition of culture nitrification by organic materials, but it should be remembered that the inhibitions observed by Winogradsky & Omeliansky (1899, 1902) and other subsequent workers may have been inhibitions not of nitrification, but of the growth of a young, and therefore sensitive, culture of nitrifying bacteria. Most of the experiments on the action of organic materials on culture nitrification have consisted in inoculating a sterile medium containing the organic material with a small amount of actively nitrifying culture, and following the subsequent formation, in the medium, of nitrite and nitrate. It is clear that any inhibition observed by this technique is as likely to be due to bacteriostasis as to inhibition of the characteristic activities of the bacteria. Meyerhof (1916 *a*, *b*, 1917) recognized the distinction, but unfortunately his results have not been given the attention they deserve. It should be mentioned

however that Nelson (1931) evaluated the glucose inhibition of both nitrite formation and nitrate formation in culture by adding glucose to actively nitrifying cultures, and found that 10% glucose inhibited the nitrification by only 4%. It is precisely this method of evaluating inhibition of nitrification, which seems the only valid one, that has been used in the present work.

SUMMARY

1. The effects of various organic materials on the oxidative nitrification process in soil have been tested by a new short-term technique.

2. None of the materials, all of which were tested at 0·1% concentration, markedly inhibited the process.

REFERENCES

Bömecke, H. (1939). *Arch. Mikrobiol.* **10**, 385.
Desai, S. V. & Fazal-ud-Din (1937). *Indian J. agric. Sci.* **7**, 895.
Fred, J. W. & Davenport, A. (1921). *Soil Sci.* **11**, 389.
Hes, J. W. (1937). Thesis: University of Groningen.
Kingma Boltjes, T. Y. (1935). *Arch. Mikrobiol.* **6**, 79.
Lees, H. (1947). *J. agric. Sci.* **37**, 27.
Lees, H. & Quastel, J. H. (1946*a*). *Biochem. J.* **40**, 803.
Lees, H. & Quastel, J. H. (1946*b*). *Biochem. J.* **40**, 815.
Lees, H. & Quastel, J. H. (1946*c*). *Biochem. J.* **40**, 824.
Meiklejohn, J. & Lees, H. (1948). In preparation.
Meyerhof, O. (1916*a*). *Pflüg. Arch. ges. Physiol.* **164**, 253.
Meyerhof, O. (1916*b*). *Pflüg. Arch. ges. Physiol.* **165**, 229.
Meyerhof, O. (1917). *Pflüg. Arch. ges. Physiol.* **166**, 240.
Nelson, D. H. (1931). *Zbl. Bakt.* II, **83**, 280.
Pandalai, K. M. (1946). *Nature, Lond.*, **158**, 484.
Winogradsky, S. (1891). *Ann. Inst. Pasteur*, **5**, 92.
Winogradsky, S. & Omeliansky, V. (1899). *Zbl. Bakt.* II, **5**, 329, 377, 429.
Winogradsky, S. & Omeliansky, V. (1902). *Arch. Sci. biol. St Petersb.* **7**, 3.

The Immobilization of Mineral Nitrogen in Soils by Different Organic Materials

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When an organic carbon source, such as glucose, is percolated through soil together with a source of mineral N, such as nitrate, a heterotrophic population quickly builds up at the expense of both compounds, and the amount of mineral N in the percolate decreases. When the supply of one compound is used up, the heterotrophic proliferation stops and the population begins to decline. The lysis of the dead cells produces ammonium ions, which are nitrified by the soil to nitrate ions, and the nitrate-N concentration begins to rise again. Not all the N initially immobilized is released again, because a certain amount is lost, as a result of its conversion to gaseous nitrogen by the denitrifying bacteria, some of which can function under aerobic conditions (Meiklejohn, 1940).

This nitrogen immobilization cycle in soil has often been studied in pots or in the field, but there seems to have been no detailed study of the biochemical processes involved. The work here reported is a preliminary survey of these processes.

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METHODS

The soil percolation technique (Lees & Quastel, 1944) has been used throughout.

Soils were percolated with either $NaNO_3$ solution alone, or with $NaNO_3$ solution plus the organic material under test, in the original soil percolation apparatus (Lees, 1946*a*). Ammonium, nitrite, and nitrate N were determined from time to time in 2 ml. samples of the percolates by the analytical methods already devised (Lees & Quastel, 1946). In fact the nitrite-N and ammonium-N concentrations in the percolates were never found to be more than a few μg./ml., and for simplicity they are excluded from the reported results. When it was necessary to use CO_2-free air, the modified percolation apparatus (Lees, 1946*a*) was employed, and air was supplied to it through two large KOH bubblers followed by a Sofnolite indicator tube. When extra CO_2 was necessary, it was introduced into the reservoir by means of a bubbler tube coupled to a Microid CO_2 generator. The CO_2 was supplied at the rate of approximately 1 bubble of CO_2/10 bubbles of entrant air.

Two soils have been used: (1) The garden soil (0·4% organic N, pH 7·8) used by Lees & Quastel (1946) (this soil is here described as the allotment soil); (2) Romney Marsh soil (0·2% organic N, pH 8·0) used by Lees (1946*b*).

RESULTS

The results of investigations on nine different organic compounds, percolated through allotment soils, are given in Table 1. The pH of the percolates rose about 0·5 during the early stages of the experiment because the immobilization of the anionic NO_3^- released cationic Na^+. In the later stages the pH again fell to normal as NO_3^- was generated. It will be seen that the initial nitrogen immobilization differed for different organic compounds. The final concentration of nitrate N, eventually built up in the percolate by lysis of the dead heterotrophs, was always less than the nitrate concentration of the nitrate-control percolate. The difference between the nitrate concentration of the control percolate and the nitrate concentration of the test percolate was presumably (at least towards the end of an experiment) some measure of denitrification loss. This loss also differed for different organic compounds. The constancy of the nitrate-N concentration of the control percolates indicated that there was no endogenous-nitrogen immobilization by compounds in the soil itself.

Table 1. *The immobilization of nitrate* N *by different organic compounds*

(Soil: allotment, 40 g. Percolate: 200 ml. 10^{-2} M-$NaNO_3$ plus the organic compound at 1·2 mg. C/ml. The minimum nitrate-N concentrations are italicized.)

Organic compound added	Nitrate N (μg./ml. percolate) after				
	4 days	8 days	12 days	20 days	24 days
None (control)	150	151	158	150	151
Glycerol	*8*	12	35	56	57
Glucose	*17*	*20*	80	93	104
Sucrose	*23*	26	81	86	103
K oleate	*20*	25	69	47	61
K stearate	*12*	20	32	50	106
None (control)	140	140	140	141	—
Na formate	102	*79*	116	135	—
Tristearin	135	130	134	*129*	—
Tributyrin	*19*	50	68	79	—
Benzoic acid	70	*60*	77	125	—

The magnitudes of the temporary immobilization of mineral nitrogen, and eventual loss of total nitrogen, are not however solely dependent on the type of carbon source. Both increase as the amount of soil in the soil-percolate system is decreased. Table 2 shows the results from an experiment in which various weights of a Romney Marsh soil were percolated with 200 ml. of 10^{-2} M-$NaNO_3$ plus glucose at 0·8 mg. C/ml. Marsh soil has good buffering properties, and no marked pH changes of the percolate were noted during the experiment. The same type of result has been obtained with allotment soil (Fig. 1) in the new percolation apparatus (Lees, 1947).

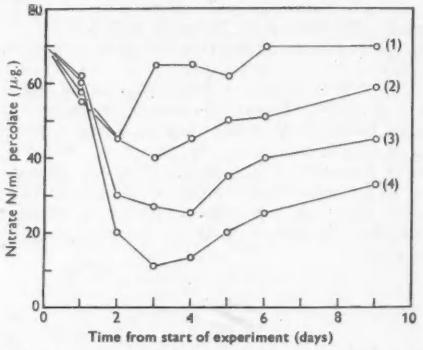

Fig. 1. The immobilization of nitrate N by various weights of allotment soil percolated with 100 ml. 10^{-2}M-$NaNO_3$ plus glucose at 0·4 mg. C/ml. Curve 1, 20 g. soil; curve 2, 10 g. soil; curve 3, 5 g. soil; curve 4, 2·5 g. soil.

The amount of nitrogen immobilized by a given amount of any one organic material seems to depend to some extent on the amount of free carbon dioxide in the soil percolate system (Table 3). Although the removal of carbon dioxide from the air entering the apparatus made no difference to the amount of nitrogen immobilized by a given amount of any one organic material (presumably because small amounts of carbon dioxide were in any case generated by the metabolic processes of the soil), the addition of carbon dioxide to the air entering the apparatus increased the amount of nitrogen immobilized.

A final point of interest is that the nitrogen-immobilizing mechanism appears to be poisoned by 10^{-3} M-azide, but not by similar concentrations of either iodoacetate or chlorate (Table 4). Un-

Table 2. *The effect of the change of soil weight on nitrogen immobilization and nitrogen loss in Romney Marsh soil percolated with nitrate and glucose*

(Percolate: 200 ml. 10^{-2}M-$NaNO_3$ + glucose at 0·8 mg. C/ml.)

Weight of soil (g.)	Nitrate N (μg./ml. percolate) after								
	1 day	2 days	3 days	4 days	5 days	6 days	9 days	13 days	16 days
2	121	92	97	100	90	71	52	32	43
10	130	114	83	65	68	49	63	59	77
20	138	95	82	75	87	85	87	91	107
40	134	94	80	80	112	108	126	126	144

Table 3. *The effect of* CO_2 *on nitrogen immobilization*

(Weight of soil: 40 g. Percolate: 200 ml. 10^{-2} M-$NaNO_3$ plus organic material at approx. 1·0 mg. C/ml.)

Soil	Organic material	Air supply	Nitrate N immobilized/mg. carbon supplied (μg.)
Allotment	None	CO_2-free + 10% CO_2	0
Allotment	Glucose	Ordinary	110
Allotment	Glucose	CO_2-free	99
Allotment	Glucose	CO_2-free + 10% CO_2	134
Allotment	Lactic acid	Ordinary	108
Allotment	Lactic acid	CO_2-free	103
Allotment	Lactic acid	CO_2-free + 10% CO_2	143
Marsh	None	CO_2-free + 10% CO_2	0
Marsh	Lactic acid	Ordinary	67
Marsh	Lactic acid	CO_2-free	70, 100, 69
Marsh	Lactic acid	CO_2-free + 10% CO_2	104, 143, 88
Marsh	Malic acid	CO_2-free	67, 48
Marsh	Malic acid	CO_2-free + 10% CO_2	100, 67
Marsh	Fumaric acid	CO_2-free	61, 51
Marsh	Fumaric acid	CO_2-free + 10% CO_2	67, 65
Marsh	Succinic acid	CO_2-free	57
Marsh	Succinic acid	CO_2-free + 10% CO_2	69

Table 4. *The effects of sodium azide, iodoacetate, and chlorate on nitrogen immobilization in allotment soil*

(Soil (40 g.) was percolated with 200 ml. 10^{-2} M-$NaNO_3$ with glucose (equivalent to 1·2 mg. C/ml.) and 10^{-3} M-azide, iodoacetate or chlorate. Control soil percolated with nitrate and glucose only. Negative control soil percolated with nitrate only.)

Poison added to percolate	Nitrate N (μg./ml. percolate) after 1 day	4 days	6 days	8 days	12 days	15 days
Na azide	144	145	134	101	115	119
Na iodoacetate	107	0	15	10	45	32
Na chlorate	140	16	30	26	54	63
Control soil	110	24	50	56	72	76
Negative control soil	158	160	156	159	159	153

published results obtained by Dr J. H. Quastel and myself have suggested that 10^{-3} M-iodoacetate or azide is toxic to nitrification in soil. Results showing the effect of 10^{-3} M-chlorate on soil nitrification have already been published (Lees & Quastel, 1945).

DISCUSSION

The results are preliminary but suggest that the immobilization of soil nitrogen by various organic materials can be studied quantitatively by a soil percolation technique.

Both nitrogen immobilization and denitrification loss have been found to vary with the type of organic compound used to bring them about. Moreover, both increase as the percolate : soil ratio is increased. Lemmermann & Wickers (1914) found, by pot experiments, that denitrification in soil was increased as the fluid : soil ratio was increased. It is possible to explain these results on the assumption that a high water content in a soil favours anaerobiosis, and therefore favours denitrification; it is not possible similarly to explain the present results. In the percolation apparatus denitrification and nitrogen immobilization were found to be greatest where the smallest weight, i.e. the shortest column, of soil was used; in other words, they were found to be greatest where anaerobiosis must have been minimal. One theory that might explain this result is that proliferation of the heterotrophs, denitrifying and other, takes place mainly in the percolate while lysis of the same heterotrophs is mainly accomplished by organisms or other agencies closely bound to the soil surface.

There are reasons for believing that nitrogen immobilization in soil may involve heavy-metal enzymes because the process is poisoned by azide. Either sulphydryl enzymes do not play any important part in nitrogen immobilization, or iodoacetate is rapidly destroyed in the soil under the experimental conditions, because iodoacetate has, if anything, a stimulatory effect on nitrogen immobilization. The chlorate-sensitive enzyme system that was found in the nitrifying bacteria by Lees & Quastel (1945) does not appear to be involved.

The effect of CO_2 in increasing nitrogen immobilization in soil is interesting because, if the increase is due to CO_2 as CO_2, and not simply to a lowering of the pH at the soil surface (where micro-organisms in general congregate), then CO_2 must be considered as one possible source of organic carbon in soil. The

work of Krampitz, Woods & Werkman (1943) is germane to this point.

It is unfortunately not possible for me to continue this work at the moment. The results are presented as provisional ones that might provide some useful pointers in future investigations on the biochemistry of nitrogen immobilization by soil.

SUMMARY

1. The immobilization of nitrogen in soils by various organic materials has been studied by a soil-percolation technique.

2. The amount of nitrogen immobilization, and the amount of denitrification, induced by an organic compound differs from compound to compound.

3. The effects of enzyme poisons and of carbon dioxide on the immobilization process suggest that some heavy-metal enzyme and some carbon dioxide-assimilative process are involved.

Most of this work was done at the Agricultural Research Council Unit of Soil Metabolism. I should like to acknowledge help and advice from Dr J. H. Quastel and to thank the A.R.C. for permission to publish these results.

REFERENCES

Krampitz, L. O., Woods, H. G. & Werkman, C. H. (1943). *J. biol. Chem.* **147**, 243.
Lees, H. (1946*a*). *Biochem. J.* **40**, 812.
Lees, H. (1946*b*). *Nature, Lond.*, **158**, 97.
Lees, H. (1947). *J. agric. Sci.* **37**, 27.
Lees, H. & Quastel, J. H. (1944). *J. Soc. Chem. Ind., Lond.*, **26**, 238.
Lees, H. & Quastel, J. H. (1945). *Nature, Lond.*, **155**, 276.
Lees, H. & Quastel, J. H. (1946). *Biochem. J.* **40**, 803.
Lemmermann, O. & Wickers, J. L. (1914). *Zbl. Bakt.* II, **41**, 608.
Meiklejohn, J. (1940). *Ann. appl. Biol.* **27**, 588.

The Effects of Zinc and Copper on Soil Nitrification

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Although many papers have been published dealing with the effects of trace elements on plants, and on soil processes in the field, there appears to have been no exhaustive study of the effect of trace elements on the microbial processes of the soil. As these processes are an essential part of agriculture, the effect of trace elements is of practical as well as theoretical interest. Hence the work here reported was begun.

METHODS

The soil-percolation technique devised by Lees & Quastel (1946*a*) for the study of soil metabolism has been used throughout. The soil-percolation apparatus used was the simplified and improved form (Lees, 1947). The influence of either zinc or copper on soil nitrification was assessed by comparing the course of nitrification in a soil percolated with $(NH_4)_2SO_4$ solution with the course of nitrification in a soil percolated with a similar $(NH_4)_2SO_4$ solution to which a known concentration of either $ZnSO_4$ or $CuSO_4$ had been added.

Mineral nitrogen analyses were performed from time to time on samples of the percolates by methods similar to those already described (Lees & Quastel, 1946*a*). As it was found early in the work that the conversion of ammonium N into nitrate N was always reasonably quantitative, ammonium-N estimations are not included in the results. Nitrite N was never found in quantities greater than a few μg./ml.; nitrite N is therefore bulked with nitrate in the reported nitrate-N figures. Percolate analyses have been converted into 'μg. N/g. soil' by the methods already described (Lees & Quastel, 1946*a*).

Copper was estimated in the percolates by the method of Sherman & McHargue (1942). Zinc was estimated spectrophotometrically.

Soils were stored damp in earthenware containers and turned from time to time. Samples of soil were taken from these containers, and air-dried and sieved (4·0–1·0 mm. mesh) before use.

All experiments were conducted with 10 g. soil percolated at 18–21° with 100 ml. of $2{\cdot}5 \times 10^{-3}$M-$(NH_4)_2SO_4$, with or without the addition of $ZnSO_4$ or $CuSO_4$. Three soils were used: (1) an allotment soil (0·4% organic N, pH 6·8); (2) a Lincolnshire Fen soil (2·5% organic N, pH 7·0); (3) a Romney Marsh soil (0·2% organic N, pH 7·8).

Stimulated soils (Lees & Quastel, 1946*b*) were prepared as follows. The soils were first percolated with 100 ml. of $2{\cdot}5 \times 10^{-3}$M-$(NH_4)_2SO_4$ until nitrification was complete, washed out two or three times with 50 ml. lots of distilled water, and finally percolated with 100 ml. distilled water for 6 hr. This wash percolate was then discarded and the soils were ready for the experiment proper.

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Poisoning of a soil by diethyldithiocarbamate was carried out as follows. Ten ml. of 4×10^{-3}M-sodium diethyldithiocarbamate were poured on to the stimulated but unwashed soil. The liquid that dripped through was recovered and again poured on to the soil. This process was thrice repeated in order to distribute the poison evenly through the soil, which was then left untouched for 1 hr. Excess of the poison was removed from the soil by three 50 ml. washings with distilled water followed by percolation for 6 hr. with 100 ml. distilled water. This wash percolate was then discarded and the soil was ready for the experiment.

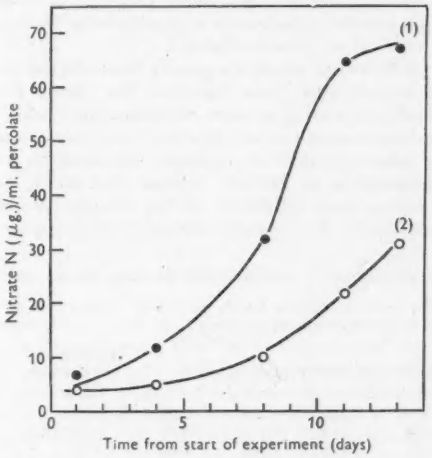

Fig. 1. The effect of zinc on nitrification in fresh (unstimulated) allotment soil. Soil (10 g.) was percolated with 100 ml. of (curve 1) $2{\cdot}5 \times 10^{-3}$M-$(NH_4)_2SO_4$ or (curve 2) $2{\cdot}5 \times 10^{-3}$M-$(NH_4)_2SO_4$ plus 4×10^{-3}M-$ZnSO_4$.

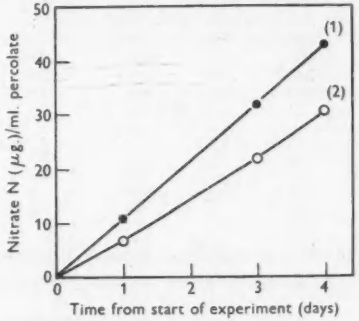

Fig. 2. The effect of zinc on nitrification in stimulated allotment soil. Soil (10 g.) was percolated with 100 ml. of (curve 1) $2{\cdot}5 \times 10^{-3}$M-$(NH_4)_2SO_4$ or (curve 2) $2{\cdot}5 \times 10^{-3}$M-$(NH_4)_2SO_4$ plus 4×10^{-3}M-$ZnSO_4$.

Experimental procedure. The toxic effect of zinc on soil nitrification may be demonstrated either as a flattening of the sigmoid curve characteristic of nitrification in a fresh soil (Fig. 1), or as a reduction of the slope of the straight line characteristic of nitrification in a stimulated soil (Fig. 2).

The inhibition due to zinc in either case may be measured as

$$100 \times \frac{C - T}{T},$$

where C = nitrate formation in absence of zinc, and T = nitrate formation in presence of zinc. This value would be constant throughout the control nitrification in ideal experiments with stimulated soils (Fig. 2), but it is less obviously so in experiments with unstimulated soils (Fig. 1).

In experiments with unstimulated soils the inhibition of nitrification due to zinc has therefore been measured at two roughly defined points during the experiment: (1) when control nitrification was half completed, and (2) when control nitrification was 90% completed. In practice the inhibition measured at point (1) has been found to agree well with that measured at point (2). But because the use of stimulated soils excludes spurious effects of zinc on the initial proliferation rate of the nitrifying organisms and minimizes the possibility of their adapting their activities to the presence of zinc, stimulated soils rather than unstimulated soils have been used in most of the work reported here.

The above explanation is based on the effect of zinc on soil nitrification; it should be understood to apply equally well to the effect of copper on the same process.

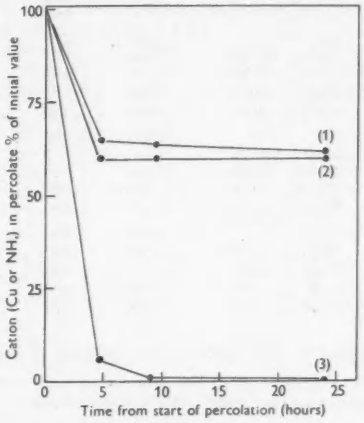

Fig. 3. The removal of ammonium and copper ions from the percolates of 10 g. Marsh soil percolated with 100 ml. of either 5×10^{-3}M-$(NH_4)_2SO_4$ or 5×10^{-3}M-$(NH_4)_2SO_4$ plus 5×10^{-3}M-$CuSO_4$. Curve 1, ammonium ion concentration in presence of copper; curve 2, ammonium ion concentration in absence of copper; curve 3, copper ion concentration.

RESULTS

When a solution containing zinc or copper is percolated through a soil, nearly all the zinc or copper is, within a few hours, transferred from the percolate to the soil. Fig. 3 illustrates the removal of copper

from copper sulphate percolated through Marsh soil, but it should be understood to typify the result obtained when a solution of either zinc or copper is percolated through any soil. The mechanism responsible for the transfer of zinc or copper from percolate to soil is not the normal soil base-exchange system; the normal base-exchange equilibrium reached by ammonium ions percolated at the same time through the same soil is unaffected by the concomitant transfer of zinc or copper to the soil. The zinc and copper are probably taken up by the organic matter of the soil (Bremner, Mann, Heintze & Lees, 1946; Brun, 1945); therefore the percolate concentrations of zinc and copper given in the tables are initial ones. Within 24 hr. from the start of any experiment reported the percolate concentration of either zinc or copper was certainly less than 0·5 μg./ml., but no exact determination of this value was carried out.

Complete protocols of experiments on the effects of zinc and copper on nitrification in unstimulated Romney Marsh soil are given in Table 1. The results from parallel experiments with stimulated Romney Marsh soil are given in Table 2.

As these two tables adequately illustrate the type of experimental data obtained, the results from experiments on stimulated allotment soil (Table 3) and stimulated Fen soil (Table 4) are given simply as mean values from replicate experiments. The results given in Tables 1–4 agree in showing that zinc was more inhibitory of soil nitrification than was copper. Low concentrations of copper appeared

Table 1. *The effects of zinc and copper sulphates on nitrification in unstimulated Romney Marsh soil*

(Soil (10 g.) was percolated with 100 ml. $2{\cdot}5 \times 10^{-3}$M-$(NH_4)_2SO_4$, with or without $ZnSO_4$ or $CuSO_4$. Results are given (1) at half completion of control nitrification; (2) at 90% completion of control nitrification.)

	Metal in percolate (M)	Nitrate N formed/g. soil in different experiments (μg.)							Inhibition of nitrification (%)
(1)	Nil	260	280	300	320	330	330	370	—
	$ZnSO_4$, 4×10^{-3}	—	—	0	90	110	—	150	74
	$ZnSO_4$, 2×10^{-3}	250	—	200	250	160	160	100	41
	$ZnSO_4$, 1×10^{-3}	110	230	250	170	320	200	230	31
	$ZnSO_4$, 5×10^{-4}	—	280	300	300	—	—	—	2
	Nil	390	370	340	340	—	—	—	—
	$CuSO_4$, 4×10^{-3}	260	160	200	—	—	—	—	44
	$CuSO_4$, 2×10^{-3}	—	260	260	200	—	—	—	31
	$CuSO_4$, 1×10^{-3}	390	350	250	260	—	—	—	13
	$CuSO_4$, 5×10^{-4}	400	350	—	—	—	—	—	1
(2)	Nil	670	590	600	700	700	700	700	—
	$ZnSO_4$, 4×10^{-3}	—	—	100	240	160	—	350	69
	$ZnSO_4$, 2×10^{-3}	500	—	550	440	500	360	270	36
	$ZnSO_4$, 1×10^{-3}	160	530	500	700	680	690	550	18
	$ZnSO_4$, 5×10^{-4}	—	590	600	700	—	—	—	0
	Nil	650	730	690	690	—	—	—	—
	$CuSO_4$, 4×10^{-3}	550	440	350	—	—	—	—	35
	$CuSO_4$, 2×10^{-3}	—	600	570	500	—	—	—	21
	$CuSO_4$, 1×10^{-3}	650	680	690	700	—	—	—	1
	$CuSO_4$, 5×10^{-4}	670	710	—	—	—	—	—	0

Table 2. *The effects of zinc and copper sulphates on nitrification in stimulated Romney Marsh soil*

(Soil (10 g.) was percolated with 100 ml. $2{\cdot}5 \times 10^{-3}$M-$(NH_4)_2SO_4$, with or without $ZnSO_4$ or $CuSO_4$. A negative inhibition means that nitrification was stimulated.)

Metal in percolate (M)	Nitrate N formed/g. soil in different experiments after 3 days (μg.)	Nitrate N formed/g. soil in different experiments after 5 days (μg.)	Inhibition of nitrification after 3 days (%)	Inhibition of nitrification after 5 days (%)
Nil	250, 270, 520, 500, 310, 310	730, 680, 700, 720, 710, 710	—	—
$ZnSO_4$, 4×10^{-3}	0, 30, 0, 100, 30, 0	0, 140, 150, 200, 0, 0	93	88
$ZnSO_4$, 2×10^{-3}	170, 140, 400, 150, 200, 140	430, 450, 500, 580, 340, 370	44	37
$ZnSO_4$, 1×10^{-3}	120, 70, 250, 370, 140, 240	400, 200, 470, 700, 580, 450	45	34
$ZnSO_4$, 5×10^{-4}	220, 240, 300, 350, 310, 300	580, 670, 720, 700, 600, 470	20	12
Nil	290, 270, 430, 410	750, 680, 600, 690	—	—
$CuSO_4$, 4×10^{-3}	250, 160, 270, 270	300, 300, 370, 440	32	48
$CuSO_4$, 2×10^{-3}	290, 140, 370, 490	740, 550, 450, 600	8	14
$CuSO_4$, 1×10^{-3}	320, 270, 410, 400	750, 620, 580, 630	0	5
$CuSO_4$, 5×10^{-4}	250, 340, 370, 480	740, 670, 590, 700	−3	1

Table 3. *The effects of zinc and copper sulphates on nitrification in stimulated allotment soil*

(Soil (10 g.) was percolated with 100 ml. $2{\cdot}5 \times 10^{-3}$M-$(NH_4)_2SO_4$, with or without $ZnSO_4$ or $CuSO_4$. Each figure is the mean value from four separate experiments. A negative inhibition means that nitrification was stimulated.)

Metal in percolate (M)	Nitrate N formed/g. soil after			Mean inhibition of nitrification after		
	1 day (μg.)	3 days (μg.)	4 days (μg.)	1 day (%)	3 days (%)	4 days (%)
Nil	120	320	440	—	—	—
$ZnSO_4$, 4×10^{-3}	40	40	90	67	87	80
$ZnSO_4$, 2×10^{-3}	80	210	270	33	34	39
$ZnSO_4$, 1×10^{-3}	80	250	320	33	22	27
$ZnSO_4$, 5×10^{-4}	95	250	350	21	22	21
$ZnSO_4$, $2{\cdot}5 \times 10^{-4}$	110	250	405	8	22	8
Nil	120	340	450	—	—	—
$CuSO_4$, 4×10^{-3}	30	80	140	75	76	69
$CuSO_4$, 2×10^{-3}	75	260	360	37	24	20
$CuSO_4$, 1×10^{-3}	100	270	380	17	21	16
$CuSO_4$, 5×10^{-4}	160	310	435	−33	9	3
$CuSO_4$, $2{\cdot}5 \times 10^{-4}$	140	370	470	−17	−9	−4

to stimulate nitrification a little, but the stimulation was not significant. Nevertheless the implied suggestion that copper may play some part in soil nitrification is supported by the results of experiments in which Fen soils were treated with sodium diethyldithiocarbamate (Table 5). This treatment,

Table 4. *The effects of zinc and copper sulphates on nitrification in stimulated Fen soil*

(Soil (10 g.) was percolated with 100 ml. $2{\cdot}5 \times 10^{-3}$M-$(NH_4)_2SO_4$, with or without $ZnSO_4$ or $CuSO_4$. Each figure is the mean value from three separate experiments. A negative inhibition means that nitrification was stimulated.)

Metal in percolate (M)	Nitrate N formed/g. soil after		Mean inhibition of nitrification after	
	2 days (μg.)	4 days (μg.)	2 days (%)	4 days (%)
Nil	240	520	—	—
$ZnSO_4$, 4×10^{-3}	150	410	37	21
$ZnSO_4$, 2×10^{-3}	180	340	25	35
$ZnSO_4$, 1×10^{-3}	180	500	25	4
$ZnSO_4$, 5×10^{-4}	230	430	4	17
$ZnSO_4$, $2{\cdot}5 \times 10^{-4}$	220	510	8	2
Nil	230	420	—	—
$CuSO_4$, 4×10^{-3}	230	340	0	19
$CuSO_4$, 2×10^{-3}	200	360	13	14
$CuSO_4$, 1×10^{-3}	230	400	0	5
$CuSO_4$, 5×10^{-4}	220	440	4	−5
$CuSO_4$, $2{\cdot}5 \times 10^{-4}$	240	440	−4	−5

which removed 13 μg. Cu/g. soil (about 30 times the exchangeable copper of the soil), effectively inhibited soil nitrification. The inhibition could be reversed completely by percolating copper sulphate through the soils, partially by percolating manganese sulphate, but not at all by percolating ferrous sulphate, zinc sulphate, or cadmium chloride.

Table 5. *The effects of various metals in reversing diethyldithiocarbamate poisoning of nitrification in Fen soil*

(Diethyldithiocarbamate-treated soils (10 g.) were percolated with 100 ml. $2{\cdot}5 \times 10^{-3}$M-$(NH_4)_2SO_4$ plus metals as indicated. Control soil was not treated with diethyldithiocarbamate.)

Metal addition to percolate (M)	Nitrate N formed/g. soil after		
	1 day (μg.)	3 days (μg.)	5 days (μg.)
Nil (control)	160	350	620
Nil	30	80	130
$CuSO_4$, 4×10^{-3}	110	360	490
$CuSO_4$, 2×10^{-3}	100	300	580
$CuSO_4$, 1×10^{-3}	80	320	580
$CuSO_4$, 5×10^{-4}	40	250	590
$ZnSO_4$, 4×10^{-3}	30	30	100
$ZnSO_4$, 2×10^{-3}	20	60	100
$CdCl_2$, 4×10^{-3}	30	40	110
$CdCl_2$, 2×10^{-3}	30	60	110
$FeSO_4$, 4×10^{-3}	20	40	140
$FeSO_4$, 2×10^{-3}	30	80	150
$MnSO_4$, 4×10^{-3}	20	120	210
$MnSO_4$, 2×10^{-3}	30	120	200

DISCUSSION

The results indicate that it should be possible to study quantitatively by the percolation technique the effect of any trace element on the microfloral activities of soil. Zinc has been found to be more inhibitory to soil nitrification than copper, but the inhibition due to either metal is less on an organic soil than on a mineral soil. This accords with the agronomic finding that trace-element deficiencies are more usually associated with organic, rather than mineral, soils. In an organic soil the availability of a trace element (and therefore its toxicity, if it is toxic) tends to be less than in a mineral soil.

It would be unwise to conclude either from the present, or from the previous results (Lees, 1946), that copper is essential to the nitrifying organisms. The balance, as well as the amounts, of trace elements in a nitrifying culture may profoundly affect the speed of nitrification therein (Meiklejohn & Lees, in preparation). The inhibition of soil nitrification that ensues when copper is removed from a soil may therefore be due, not directly to a copper deficiency, but to a resultant imbalance of the remaining available trace elements. Such a theory would perhaps explain the partial reversal of diethyldithiocarbamate poisoning by manganese. Lucas (1945) has shown by plant growth experiments that there is some interaction between soil copper and soil manganese.

The results of the present work suggest a possible basis for a direct biological test for trace element deficiencies in soil. A diethyldithiocarbamate-treated soil is copper-deficient; as a result of this deficiency the nitrifying power of the soil is lowered, but may be raised again if copper is added to the soil. Copper deficiency in soil is therefore biologically detectable by the fact that addition of copper to the soil increases the nitrifying power. The possibility of measuring natural trace-element deficiencies by this sort of technique, which is an extension of the culture test elaborated by Mülder (1940), is obvious.

SUMMARY

1. It has been found possible quantitatively to study the effects of zinc and copper on soil nitrification by the percolation technique.

2. The toxic effect of either element is less on an organic than on a mineral soil.

3. Sodium diethyldithiocarbamate poisons soil nitrification. The poisoning is reversible by copper and partially reversible by manganese.

4. The possible importance of copper in nitrification is discussed.

5. It is suggested that the results might form the basis of a direct soil test for trace-element deficiencies.

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REFERENCES

Bremner, J. M., Mann, P. J. G., Heintze, S. G. & Lees, H. (1946). *Nature, Lond.*, **158**, 790.
Brun, T. S. (1945). *Bergens Mus. Aarb.* **6**, 221.
Lees, H. (1946). *Nature, Lond.*, **158**, 97.
Lees, H. (1947). *J. agric. Sci.* **37**, 27.
Lees, H. & Quastel, J. H. (1946*a*). *Biochem. J.* **40**, 803.
Lees, H. & Quastel, J. H. (1946*b*). *Biochem. J.* **40**, 824.
Lucas, R. E. (1945). *Proc. Soil Soc. Amer.* **10**, 269.
Mülder, E. G. (1940). *Antonie v. Leeuwen.* **6**, 99.
Sherman, G. D. & McHargue, J. S. (1942). *J. Ass. off. agric. Chem., Wash.*, **25**, 510.

Studies in Detoxication

16. THE METABOLISM OF ACETANILIDE IN THE RABBIT

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It is now sixty years since Cahn & Hepp (1887) introduced acetanilide (antifebrin) as an analgesic and antipyretic. Nevertheless, its fate in the body has not been studied quantitatively until recently (Greenberg & Lester, 1946). Furthermore, its metabolites are not exactly known. Early work (Müller, 1887; Kumagawa, 1888; Gregoire & Hendrick, 1904, etc.) established that acetanilide was oxidized *in vivo*, and that by suitable treatment of the urine *p*-aminophenol could be isolated. Jaffé & Hilbert (1888) found that the metabolites of acetanilide in the dog were different from those in the rabbit. From dog urine the main compound isolated was benzoxazolone together with small amounts of *p*-aminophenol, whereas from rabbit urine only *p*-aminophenol was isolated. It is likely that the benzoxazolone of Jaffé & Hilbert (1888) is an artefact derived from *o*-aminophenol and urea. It appears therefore that in dogs acetanilide is oxidized mainly in the *o*-position and in rabbits in the *p*-position. None of the above-mentioned work gives any clue whether acetanilide is deacetylated or not.

In 1889 Mörner, carefully avoiding hydrolytic procedures, worked up urine from a subject to whom acetanilide had been administered, and isolated potassium *p*-acetamidophenylsulphate as a double

salt with potassium ethyl oxalate. He also isolated a glucuronide which he was unable to characterize, but thought to be *p*-acetamidophenylglucuronide. Mörner's work suggests that the metabolites of acetanilide in man are acetylated.

From a study of the pharmacological properties of acetanilide and a number of related compounds, Hinsberg & Treupel (1894) concluded that the antipyretic and analgesic properties of the acetanilide group of drugs were related to their conversion in the organism to *p*-aminophenol or *p*-acetamidophenol. They found both these phenols to have a prompt antipyretic action in man (*p*-acetamidophenol is a more powerful antipyretic than phenacetin), but the action was not prolonged enough for successful use in therapy. It therefore appeared that the activity of acetanilide or phenacetin could be explained by its slow conversion *in vivo* into one or other of these phenols. Hinsberg & Treupel (1894) favoured *p*-acetamidophenol. More recently, Michel, Bernheim & Bernheim (1937) have shown that acetanilide is split into aniline and acetic acid by rat liver and kidney tissue, and that aniline can be oxidized by the same tissues to *p*-aminophenol. From this it appears possible that acetanilide might be deacetylated and then oxidized to *p*-aminophenol.

The quantitative work of Greenberg & Lester (1946) on the fate of acetanilide in man has shown that 70–90 % of the dose is excreted as conjugated *p*-aminophenol, 96 % as *O* conjugates (i.e. glucuronide and ethereal sulphates) and 4 % as *N* conjugates (probably *p*-acetamidophenol). Other metabolites of acetanilide have been postulated by various workers, e.g. *N*-acetyl-*N*-phenylhydroxylamine (Ellinger, 1920) and certain azo compounds (Greenberg & Lester, 1946), but there appears to be no real basis for the existence of these metabolites.

From the work discussed it is therefore clear that the exact nature of the metabolites of acetanilide is not known. In the present paper evidence will be presented to show that, in the rabbit, the main metabolites are the glucuronide and ethereal sulphate of *p*-acetamidophenol. Other work in progress in this laboratory indicates that acetanilide and aniline are not metabolically equivalent as is sometimes thought, for the metabolites of aniline are not the same as those of acetanilide.

METHODS

Animals. Rabbits fed on 50 g. Lever's cubes daily were used throughout the work. Compounds were administered by stomach tube with water.

Glucuronic acid in urine was determined by the method of Hanson, Mills & Williams (1944).

Ethereal sulphate was determined by Folin's gravimetric method.

EXPERIMENTAL AND RESULTS

Table 1 indicates that the ratio glucuronide/ethereal sulphate (G/E) for acetanilide (5·8) is similar to that for *p*-acetamidophenol (6·3) and different from that for *p*-aminophenol (2·5). These figures could be interpreted as meaning that acetanilide is first oxidized to *p*-acetamidophenol which then conjugates with glucuronic and sulphuric acids in the same ratio as though the phenol itself were fed. The G/E ratio for *p*-aminophenol is so different from that of acetanilide that it could hardly be expected that acetanilide is transformed to *p*-aminophenol, i.e. via aniline. That this interpretation is correct is supported by the isolation experiments described below. The sum of the glucuronic acid and ethereal sulphate outputs ($G+E$) shows that 82 % of the acetanilide can be accounted for. $G+E$ is somewhat less for *p*-acetamidophenol (73 %) and for *p*-aminophenol (60·5 %). This lesser recovery of the phenols can be accounted for by the fact that they are rapidly absorbed and excreted and some appear in the free state in the urine. In the case of acetanilide there is a slow conversion to the phenol, which therefore becomes more completely conjugated. Here we have an illustration of the time factor in detoxication.

Table 1. *The output of glucuronic acid and ethereal sulphate in rabbits receiving orally acetanilide and related compounds*

Compound	Dose (g./kg.)	% of dose excreted as: Glucuronide* (G)	Ethereal* sulphate (E)	$G+E$	G/E
Acetanilide	0·25	70	12	82	5·8
p-Acetamidophenol	0·28	63	10	73	6·3
p-Aminophenol	0·25	43	17·5	60·5	2·5

* Average results.

The effect of acetanilide on rabbits. In all experiments with rabbits receiving oral doses of acetanilide of 0·5 g./kg. it was observed that the animals fell into a deep narcosis for 4–5 hr. The animals all recovered without any apparent deleterious effect. This narcosis was not observed when the animals received *p*-acetamidophenol or *p*-aminophenol.

ISOLATION OF METABOLITES FROM ACETANILIDE URINE

(*a*) *Nature of the urine.* Urine from rabbits receiving oral doses of 0·5 g./kg. of acetanilide does not reduce Benedict's reagent except on prolonged boiling. It does not reduce cold ammoniacal $AgNO_3$. The urine does not give a colour with $FeCl_3$, nor does an ether extract of the untreated urine. The urine gives an intense naphthoresorcinol reaction for glucuronic acid and is laevorotatory.

(*b*) *Extraction at alkaline* pH. 2 g. of acetanilide were fed to each of 5 rabbits and the urine (1 l.) collected during the next 24 hr. A few ml. of 2N-KOH were added to the urine, which was then extracted continuously with ether for 1 hr. The extract on evaporation left a small oily residue which partly crystallized. A few drops of 2N-HCl were added to the residue and the whole was filtered, leaving a crystalline mass (40 mg.), which on recrystallization from hot water, was identified as acetanilide (m.p. and mixed m.p. 112–113°). The filtrate from these crystals was now made alkaline and extracted with ether. The ether was evaporated and the residue benzoylated with benzoyl chloride and NaOH. From the mixture there were isolated 20 mg. of benzanilide (m.p. and mixed m.p. 160°). The yields of aniline and acetanilide in four experiments are given in Table 2. Aminophenols were not found in this extract.

Table 2. *Recovery of acetanilide and aniline from the urine of rabbits receiving acetanilide orally*

	% of dose recovered as	
Exp.	Acetanilide	Aniline
1	0	0·5
2	0·4	0·1
3	0·2	0·1
4	0·2	0

(*c*) *Extraction at acid* pH. The above urine was made acid to congo red with HCl and extracted with ether for 1 hr. A small tarry residue remained on evaporation of the ether, but this contained nothing of significance.

(*d*) *Mild hydrolysis of acetanilide urine.* A total of 6 g. of acetanilide was fed to three rabbits and the urine (500 ml.) was collected during the following 24 hr. 350 ml. of the urine were made alkaline with a few ml. of 2N-KOH and then extracted continuously for 5 hr. with ether. Evaporation of the ether left 10 mg. of acetanilide; no aniline was found. The extracted urine was now made normal with respect to HCl by the addition of conc. HCl and then boiled gently for 20 min. A separate experiment showed that *p*-aminophenylglucuronide was only slightly hydrolyzed under these conditions. The urine was now cooled and made slightly alkaline with solid Na_2CO_3 and then extracted for 1 hr. with ether. On cooling the ether extract, 200 mg. of *p*-aminophenol separated (m.p. 184° after recrystallization). The ether was now taken to 5 ml. and a further 350 mg. of *p*-aminophenol were recovered. The filtrate from the second crop of crystals was now evaporated to a paste and the solid matter separated and benzoylated. It yielded 20 mg. of *ON*-dibenzoyl-*p*-aminophenol (m.p. 229–230°). The mother liquor of the above paste was now stirred with 2N-NaOH and then extracted with ether. The ether yielded nothing. The alkaline liquor was made less alkaline by acidifying and then adding Na_2CO_3. Extraction with ether yielded a small residue, which on benzoylation yielded 1 mg. of dibenzoyl-*p*-aminophenol (m.p. 225–230°). No trace of *o*-aminophenol was found (*ON*-dibenzoyl-*o*-aminophenol has m.p. 180°). Further extraction of the urine with ether for 8 hr. yielded a small amount of *p*-aminophenol.

The total amount of *p*-aminophenol obtained by this mild hydrolysis was equivalent to 11% of the acetanilide fed. Quantitative determinations of the ethereal-sulphate output showed that 12% of acetanilide was excreted as a sulphate (see Table 1). It is therefore concluded that the ethereal-sulphate fraction of acetanilide urine contains no other phenol than *p*-aminophenol. Since very little of free amino compounds is excreted in acetanilide urine the actual phenol excreted as a sulphate is *p*-acetamidophenol (cf. Mörner, 1889).

(*e*) *The glucuronide fraction.* (1) *Detection of p-aminophenol.* Two rabbits were each fed 2 g. of acetanilide and the urine collected for 24 hr. The urine was made faintly acid with acetic acid and treated with saturated normal lead acetate. The precipitate was removed and discarded. The filtrate was made faintly alkaline and basic lead acetate added until precipitation was complete. The basic lead precipitate was filtered and washed with water. It was then suspended in water and the lead removed with H_2S. After removal of the PbS, the filtrate was evaporated with addition of ethanol *in vacuo* at 40–50° to a thin syrup. A small amount of inorganic material was removed from this syrup by diluting with ethanol and filtering. On addition of ether or chloroform to the ethanolic solution, the glucuronide was thrown down as amorphous hygroscopic flocks. In four separate experiments this material could not be induced to crystallize. The glucuronide gum gave only a very feeble diazo reaction indicating the almost total absence of free aromatic amino groups. On keeping a small portion of the gum with conc. HCl at room temperature there was no change in the diazo reaction.

The gum obtained after the feeding of 4 g. of acetanilide was now boiled for 1 hr. with 100 ml. 5N-HCl. The resulting solution gave an intense *p*-aminophenol type of diazo reaction (i.e. on diazotizing and coupling with dimethyl-α-naphthylamine there is obtained at first a fleeting dirty blue colour and then, on standing, the solution becomes a deep blue colour). The solution was made just alkaline and extracted with ether in a separatory funnel. The ether extract was evaporated and the residue benzoylated. There were obtained 800 mg. of *ON*-dibenzoyl-*p*-aminophenol (m.p. and mixed m.p. 234°) after recrystallization from ethanol. The yield was 5% of the dose of acetanilide.

(2) *Acetylation and methylation of the glucuronide gum.* The gum from the urine of rabbits after the feeding of 10 g. of acetanilide was divided into two equal parts. The first part was methylated with methyl sulphate and alkali followed by methyl iodide and Ag_2O. The product was an intractable chloroform-soluble gum which was not further studied.

The second part was dissolved in 100 ml. of a 50 : 50 pyridine-acetic anhydride mixture and left overnight at room temperature. The mixture was then poured into 1 l. of dilute HCl, and the solution extracted with chloroform.

The chloroform extract was dried with anhyd. $CaCl_2$ and evaporated on a water bath. From the residue there were obtained 600 mg. (3·5% of the dose) of a white amorphous solid. This solid recrystallized from acetone water as needles, m.p. 224° (decomp.). It was acid to litmus and gave the naphthoresorcinol reaction on prolonged boiling. The diazo reaction was negative, but was positive after hydrolysis. It was laevorotatory, $[\alpha]_D^{20°} = -15·1°$ (*c*, 2·8 in acetone). This compound appears to be *p-acetamidophenyltriacetylglucuronic acid*. (Found: C, 52·7; H, 5·2; N, 3·7%. $C_{20}H_{23}O_{11}N$ requires C, 53·0; H, 5·1; N, 3·1%.) (Ultraviolet absorption spectrum: $\lambda_{max.}$ 245 mμ.; $\epsilon_{max.}$ 18,000 in ethanol (see Fig. 1).)

300 mg. of the above acid dissolved in acetone were methylated with methyl iodide and Ag_2O at room temperature overnight. Removal of the solvent yielded an *ester* (210 mg.) which crystallized from ethanol in small sheaves of rods with m.p. 210° and showing $[\alpha]_D^{25°} = -22·5°$ (*c*, 3·3 in chloroform). (Found: C, 54·05; H, 5·55; N, 3·4%. $C_{21}H_{25}O_{11}N$ requires C, 54·0; H, 5·4; N, 3·0%.) Its ultraviolet absorption spectrum in ethanol (see Fig. 1) was determined, and was found to contain a band with $\lambda_{max.}$ at 246 mμ.; $\epsilon_{max.}$ 15,600. It was neutral in reaction, sparingly soluble in water, but easily soluble in acetone and ethanol. It was identified as the *methyl ester* of *p-acetamidophenyltriacetyl-β-D-glucuronic acid*, for it did not depress the melting point of an authentic sample of this ester and was identical with it in all respects (see below).

(3) *Isolation of the benzylamine salt of p-acetamidophenylglucuronide.* Identification of the glucuronide of acetanilide urine was finally established through the use of benzylamine. It was found that under certain conditions aminophenylglucuronides could be isolated from solutions in almost quantitative yields as crystalline benzylamine salts. This observation was made after testing a large number of bases including brucine, *o*-toluidine, benzidine, aniline, benzylthiourea and guanidine.

A rabbit was given 2 g. of acetanilide orally and its urine (225 ml.) was collected during the next 24 hr. This urine was estimated to contain about 1 g. of glucuronic acid more than normal. The glucuronide gum was prepared by systematic lead precipitation as already described. The gum was freed from small amounts of inorganic material by dissolution in ethanol and filtering. The purified gum was now dissolved in sufficient aqueous ethanol to produce a 5% solution of the gum. To this solution was added slightly more than one equivalent of benzylamine. Then ethyl acetate and ethanol were added alternately so that the gum concentration was 0·1–0·5% and enough ethyl acetate had been added to make the solution cloudy. The mixture was then allowed to stand at 0° for a few days. The crystalline precipitate (1·03 g., m.p. 196–197°) was filtered off and washed with ethanol. The filtrate was taken down and then diluted with ethanol-ethyl acetate as before. On standing a further 0·35 g. of crystals (m.p. 195–196°) was obtained. Repetition of the process yielded another 0·21 g. The total material recovered was 1·59 g. or 71% of the extra glucuronic acid in the urine. The crude salt gave a very faint diazo reaction which disappeared after one recrystallization from 95% ethanol. The salt showed $[\alpha]_D^{25°} = -63·8°$ (*c*, 4·7 in water), m.p. 195–197° and mixed m.p. 195–197° with authentic benzylamine *p*-acetamidophenylglucuronate (see below). (Found: C, 55·3; H, 6·2; N, 6·5%. $C_{21}H_{26}O_8N_2.H_2O$ requires C, 55·7; H, 6·2; N, 6·2%.)

PREPARATION OF REFERENCE COMPOUNDS

Benzylamine salt of p-acetamidophenylglucuronide. Four rabbits were each fed 1·5 g. of *p*-acetamidophenol by stomach tube. This phenol is rapidly conjugated and excreted and therefore the urine was collected for 3–4 hr. after feeding. The basic lead acetate precipitate was obtained in the usual manner and the Pb removed by H_2S. The filtrate from the PbS gave, on concentration, *p*-acetamidophenylglucuronide as a gum which could not be crystallized. (Crystalline *p*-acetamidophenylglucuronide was obtained by acetylation of *p*-aminophenylglucuronide (see below).) The gum was dissolved in 3 vol. of ethanol and benzylamine and a mixture of ethyl acetate and ethanol was added to give a cloudy solution with a concentration of 0·1% as regards the gum. On standing at 0° the *benzylamine salt* crystallized out as needles (yield, 0·5 g.). The salt was recrystallized from water-ethanol-ethyl acetate mixtures, and had m.p. 195–197° and $[\alpha]_D^{25°}$, $= -64·1°$ (*c*, 4·4 in water). (Found: C 55·9; H, 6·3; N, 6·4%. $C_{21}H_{26}O_8N_2.H_2O$ requires C, 55·7; H, 6·2; N, 6·2%.) It was very soluble in water but insoluble in dry organic solvents.

Benzylamine salt of p-aminophenylglucuronide. *p*-Aminophenylglucuronide (m.p. 215–216°; $[\alpha]_D = -82·7°$ in dilute HCl) was prepared according to Williams (1943). From the urine collected during the first 3 hr. after feeding 10 g. of *p*-aminophenol, 3·3 g. of the glucuronide were isolated. For the 24 hr. urine the yield of crystalline glucuronide was 3·95 g. or 13% of the dose. To prepare the benzylamine salt, the glucuronide was suspended in a small amount of water and slight excess of benzylamine added. This was followed by 5 vol. ethanol and then ethyl acetate until the solution became cloudy. On standing at 0°, the *benzylamine salt* of *p-aminophenylglucuronide* separated as square plates. This was recrystallized from the same solvents, and had m.p. 211–212° and $[\alpha]_D^{20°} = -62·7°$ (*c*, 6·5 in 0·2N-HCl). (Found: C, 53·1; H, 6·6; N, 6·4%. $C_{19}H_{24}O_7N_2.2H_2O$ requires C, 53·3; H, 6·6; N, 6·5%.)

p-Acetamidophenylglucuronide. 2 g. of *p*-aminophenylglucuronide were dissolved in excess dilute Na_2CO_3 solution and shaken with an excess of acetic anhydride until tests for free aromatic NH_2 groups were negative. The *N*-acetylated glucuronide was obtained from the solution as the insoluble lead salt by addition of basic lead acetate solution. The lead salt, suspended in water, was freed from Pb by H_2S. The filtrate from the PbS was concentrated *in vacuo* to a clear syrupy solution (5–10 ml.). To this were added 100 ml. ethanol and the mixture was concentrated *in vacuo* at 40° to 5 ml. 50 ml. ethanol were now added followed by enough ether to give a slight precipitate. The whole was now kept at 0° for 48 hr. whereby *p-acetamidophenylglucuronide* (0·7 g.) crystallized as long, narrow plates. It was recrystallized from hot 95% ethanol (m.p. 193°, decomp. after sintering at 100°); $[\alpha]_D^{22°} = -63·9°$ (*c*, 7·5 in water). (Found: C, 45·3; H, 6·2; N, 3·95%. $C_{14}H_{17}O_8N.2\frac{1}{2}H_2O$ requires C, 45·2; H, 6·0; N, 3·8%.) It was very soluble in water, soluble in ethanol and insoluble in ether. It did not convert haemoglobin to methaemoglobin *in vitro* at 37° (cf. Williams, 1943).

p-Acetamidophenyltriacetylglucuronic acid methyl ester. 300 mg. of *p*-acetamidophenylglucuronide were dissolved in 50 ml. of absolute ethanol and an ethereal solution of

diazomethane (prepared from 1 g. of nitrosomethylurea) added. After 3 hr. the yellow solution was filtered and evaporated *in vacuo* leaving a white, amorphous, hygroscopic solid which was neutral to litmus. This was dissolved in 5 ml. of pyridine and then 5 ml. of acetic anhydride were added. After standing overnight, the mixture was diluted with 50 ml. of water and cooled to 0° for 2 hr. A mass of silky white needles (190 mg.) was deposited. A further 80 mg. were obtained by extracting the mother liquor with chloroform, and crystallizing from water. The product at this stage was apparently a *hydrate* for it melted at 100°, and then resolidified to a glass which melted indefinitely at 150–200°. Drying at 110° in air led to some decomposition because the material turned brown. The anhydrous product was obtained by dissolving the hydrate in absolute ethanol and scratching the beaker, whereby the *methyl ester* of *p-acetamidophenyltriacetylglucuronide* crystallized as sheaves of short needles, m.p. 200–205° and showing $[\alpha]_D^{25°} = -22{\cdot}1°$ (*c*, 7 in chloroform). (Found: C, 54·5; H, 5·6; N, 2·9%. $C_{21}H_{25}O_{11}N$ requires C, 54·0; H, 5·4; N, 3·0%.) Ultraviolet absorption spectrum, $\lambda_{max.}$ 246 mμ., with ϵ_{max} 15,600. The compound is very soluble in acetone, ethanol and chloroform, but sparingly soluble in water and ether.

Benzylamine salt of o-aminophenylglucuronide. *o*-Aminophenylglucuronide (decomp. at 300°; $[\alpha]_D = -76{\cdot}1°$ in dilute HCl) was prepared by feeding rabbits with *o*-aminophenol according to Williams (1943). The *benzylamine salt* was prepared as for the *p*-isomer. The salt formed clumps of short needles, m.p. 204–205° and $[\alpha]_D^{20°} = -61{\cdot}3°$ (*c*, 6·1 in water). (Found: C, 58·0; H, 6·1; N, 6·9%. $C_{19}H_{24}O_7N_2$ requires C, 58·2; H, 6·1; N, 7·1%.)

SPECTROSCOPIC OBSERVATIONS

The ultraviolet absorption spectra of *p*-aminophenylglucuronide and its acetyl derivatives and *p*-acetamidophenol are recorded in Fig. 1. From this figure it can be seen that derivatives of *p*-aminophenol can be readily distinguished from those of *p*-acetamidophenol. Whereas the spectra of *p*-aminophenol (see Williams, 1947; Morton & Stubbs, 1940) and *p*-aminophenylglucuronide show two maxima in the region 220–320 mμ., with molecular extinction coefficients less than 10,000, all the derivatives of *p*-acetamidophenol exhibit only one band in this region with an extinction coefficient greater than 10,000. *p*-Acetamidophenol itself shows a band at $\lambda_{max.}$ 250 mμ., $\epsilon_{max.}$ 13,800 and an inflexion at 285 mμ., ϵ 3500, whereas *p*-aminophenol shows two bands at $\lambda_{max.}$ 234 and 395 mμ. with $\epsilon_{max.}$ 7950 and 2700 respectively. For the glucuronides, the figures are: *p*-acetamidophenylglucuronide, $\lambda_{max.}$ 243 mμ., $\epsilon_{max.}$ 10,800 and *p*-aminophenylglucuronide, $\lambda_{max.}$ 231 and 291 mμ., $\epsilon_{max.}$ 8700 and 1600.

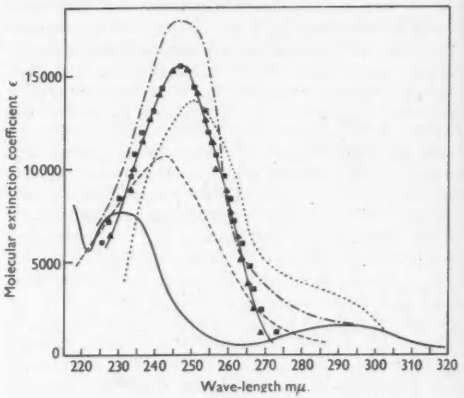
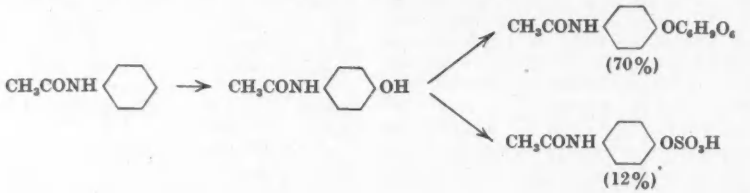


Fig. 1. Ultraviolet absorption spectra of *p*-aminophenylglucuronide and related compounds. · · · · *p*-acetamidophenol in ethanol: $\lambda_{max.}$ 250 mμ., $\epsilon_{max.}$ 13,800; ~ 285 mμ., ϵ 3500. - - - - - *p*-acetamidophenylglucuronide in water: $\lambda_{max.}$ 243 mμ., $\epsilon_{max.}$ 10,800. –·–·– *p*-acetamidophenyltriacetylglucuronic acid in ethanol: $\lambda_{max.}$ 245 mμ., $\epsilon_{max.}$ 18,000. ■—■ *p*-acetamidophenyltriacetylglucuronide methyl ester (synthetic) in ethanol: $\lambda_{max.}$ 246 mμ., $\epsilon_{max.}$ 15,600. ▲—▲ *p*-acetamidophenyltriacetylglucuronide methyl ester (from acetanilide urine) in ethanol: $\lambda_{max.}$ 246 mμ., $\epsilon_{max.}$ 15,600. —— *p*-aminophenylglucuronide in 0·1 N-KOH: $\lambda_{max.}$ 231 and 291 mμ., $\epsilon_{max.}$ 8700 and 1600.

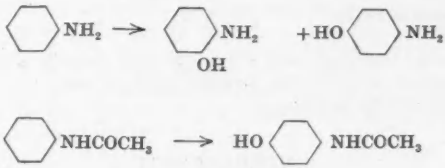
DISCUSSION

The present work proves that the metabolites of acetanilide in the rabbit are *p*-acetamidophenylglucuronide and *p*-acetamidophenylsulphuric acid which are excreted in the ratio of about 6 to 1 and account for over 80% of the acetanilide fed. Small traces of acetanilide (*c*. 0·2% of dose) and of aniline (*c*. 0·2%) are also found in the urine. The metabolism of acetanilide in the rabbit can therefore be represented as follows:



Acetanilide undergoes very little deacetylation (less than 5% of the dose as determined by diazotization and coupling), an observation which agrees with our earlier results on *p*-substituted acetanilides (Smith & Williams, 1948). Michel *et al.* (1937) have shown that acetanilide is deacetylated by isolated rat tissues. Our results indicate that in the intact rabbit either such a deacetylating system does not occur or, if it does, it has no opportunity of functioning to any great extent. Some animals may, however, be able to deacetylate the aromatic acetamido group, for Sykes (1944) has shown that whereas N^4-acetylsulphamezathine is excreted almost entirely unchanged by man, 40% is deacetylated in the pigeon. Whether or not an acetamido compound is deacetylated *in vivo* may have an important bearing on its fate in the body, as will be seen from what follows.

We have found (Smith & Williams, unpublished) that in rabbits aniline is oxidized but is not acetylated, and the products of oxidation are *o*- and *p*-aminophenols (together with small amounts of what we think is 2:4-dihydroxyaniline). No trace of *o*-aminophenol or its *N*-acetyl derivative was detected in acetanilide urine:



Present views (see Pauling, 1945) concerning the effect of substituents on the activity of aromatic rings in substitution reactions indicate that the amino group in aniline activates, mainly through resonance, the ortho and para positions. It is to be expected therefore that biological oxidation will take place in these positions. This actually occurs with aniline in the rabbit. In the case of acetanilide, biological oxidation occurs only in the para position. This result is similar to the behaviour of acetanilide on nitration which gives rise to the formation of 95% *p*- and 5% *o*-nitroacetanilide, whereas nitration of aniline gives 50% *p*- and 40% *o*-nitro compound (Cumming, Hopper & Wheeler, 1937). Now acetylation of the amino group of aniline will result in some deactivation of the ring. This deactivation is more pronounced in the *o*-position than in the *p*-position through the so-called inductive effect of the acetamido group working in opposition to the resonance effect. In addition to this, the acetamido group is larger than the amino group and will therefore exert a steric hindrance effect on ortho substitution. From these arguments it can be seen that ortho substitution of acetanilide may be hindered both by steric hindrance and by deactivation through induction. For biological oxidation to take place, it may be assumed that a minimum activation of the position to be oxidized is necessary. The absence of biological oxidation of acetanilide in the ortho position could therefore be explained on the grounds that (1) the ortho positions are not sufficiently activated for biological oxidation, and (2) steric hindrance due to the acetamino group prevents the approach of the biological oxidizing system to the ortho positions. Oxidation of acetanilide should therefore take place in the para position, for here there is no steric hindrance and the position is sufficiently activated, through resonance, for the enzyme system to attack it.

It follows from the above arguments that if an animal can deacetylate acetanilide to aniline, then it is to be expected that acetanilide will give rise to *o*- and *p*-aminophenol in such an animal. When acetanilide is fed to dogs, both *o*- and *p*-aminophenol can be detected in the urine (Jaffé & Hilbert, 1888). It is probable that the dog possesses an active deacetylating enzyme for aromatic acetamido compounds.

The fate of acetanilide in man (cf. Mörner, 1889) is probably very similar to that in the rabbit. Lester & Greenberg (1947) have recently shown that the major metabolites of acetanilide in man are *p*-acetamidophenol and its *O* conjugates, although they did not isolate these compounds.

SUMMARY

1. It has been shown that, in the rabbit, acetanilide is oxidized almost entirely to *p*-acetamidophenol, which is excreted in the urine as *p*-acetamidophenylglucuronide and *p*-acetamidophenylsulphuric acid, these occurring in the ratio 6 : 1.

2. Less than 5% of the acetanilide fed is deacetylated and only traces of aniline and unchanged acetanilide were isolated.

3. *p*-Acetamidophenylglucuronide can be conveniently isolated as the benzylamine salt.

4. The following reference compounds have been synthesized: the benzylamine salts of *o*- and *p*-aminophenylglucuronides and of *p*-acetamidophenylglucuronide; *p*-acetamidophenylglucuronide and the triacetyl derivative of its methyl ester.

5. The ultraviolet absorption spectra of the compounds studied have been recorded and discussed.

6. The theoretical implications of the biological oxidation of acetanilide have also been discussed.

REFERENCES

Cahn, J. & Hepp, P. (1887). *Berl. klin. Wschr.* **24**, 4, 26.
Cumming, W. M., Hopper, I. V. & Wheeler, T. S. (1937). *Systematic Organic Chemistry*, 3rd ed. p. 270. London: Constable.
Ellinger, P. (1920). *Hoppe-Seyl. Z.* **111**, 86.
Greenberg, L. E. & Lester, D. (1946). *J. Pharmacol.* **88**, 87.
Gregoire, A. & Hendrick, J. (1904). *Bull. Soc. Chim. Belg.* **18**, 94.
Hanson, S. W. F., Mills, G. T. & Williams, R. T. (1944). *Biochem. J.* **38**, 274.
Hinsberg, O. & Treupel, G. (1894). *Arch. exp. Path. Pharmak.* **33**, 216.
Jaffé, M. & Hilbert, P. (1888). *Hoppe-Seyl. Z.* **12**, 295.
Kumagawa, M. (1888). *Virchows Arch.* **113**, 135.
Lester, D. & Greenberg, L. E. (1947). *J. Pharmacol.* **90**, 68.
Michel, H. O., Bernheim, M. L. C. & Bernheim, F. (1937). *J. Pharmacol.* **61**, 321.
Mörner, K. A. H. (1889). *Hoppe-Seyl. Z.* **13**, 12.
Morton, R. A. & Stubbs, A. L. (1940). *J. chem. Soc.* p. 1349.
Müller, F. (1887). *Dtsch. med. Wschr.* **13**, 27.
Pauling, L. (1945). *Nature of the Chemical Bond*, pp. 147–52. London and New York: Cornell Univ. Press.
Smith, J. N. & Williams, R. T. (1948). *Biochem. J.* **42**, 351.
Sykes, W. O. (1944). *Biochem. J.* **38**, xxix.
Williams, R. T. (1943). *Biochem. J.* **37**, 329.
Williams, R. T. (1947). *Biochem. J.* **41**, 1.

The Molecular Weight of Insulin and its Dependence upon pH, Concentration and Temperature

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In a previous paper (Gutfreund, 1948) it was shown from the results of osmotic and diffusion-sedimentation measurements that the molecular weight of insulin is 47,000–48,000 in solutions of pH 7·0 containing 0·5–1·0% protein. Molecules of that size were found to dissociate into smaller units on dilution to protein concentrations of 0·2% or less. Sjögren & Svedberg (1931) have determined the sedimentation constant of insulin over a range of pH from 3·5 to 12·3 and found from their data that the molecular weight has a maximum at pH 6·8. They concluded that insulin dissociates both in acid and in alkaline solutions and also that this dissociation is reversible if the solution is not kept too long at a pH outside the stability region. Gutfreund & Ogston (1946) re-examined the sedimentation constant over a range of pH from 3·5 to 9·6 and found that the sedimentation velocity of the main boundary remained constant between pH 6·7 and 9·6. In some cases, however, some slower-sedimenting material was present.

Gutfreund & Ogston were interested in the sedimentation rate of the main boundary. A re-examination of the sedimentation diagrams showed the presence, in solutions more alkaline than pH 8, of some more slowly sedimenting material which did not form a clear boundary.

The recent work of Waugh (1944, 1945, 1946) showed that insulin undergoes drastic and reversible changes in acid solutions, and the studies of the influence of pH on the properties of insulin solutions have therefore been continued and extended by means of both the osmotic pressure and ultra centrifugal technique.

EXPERIMENTAL

The sedimentation constants were determined at Oxford in the oil-turbine ultracentrifuge (Svedberg type). The sedimenting boundaries were observed by the Philpot (1938) method. The speed of the centrifuge during all the experiments was about 1000 rev./sec. A detailed description of the accuracy of the measurements is given by Gutfreund & Ogston (1946). All values of sedimentation constants (S_{20}) recorded are corrected to sedimentation in water at 20°.

For the osmotic-pressure measurements an apparatus of the type described by Adair (1925) was used. The pressure was measured directly as the height of solution in the capillary tube. The protein concentration of the equilibrated solution was calculated from Δn (refractive index difference between buffer and solution) assuming a specific refractive increment of 180×10^{-5}. Δn was measured in a Zeiss interferometer, the usual type of refractometers not being accurate enough for measurements on the very dilute solutions used throughout this work.

Since the solutions were very dilute all the values for molecular weights, recorded in this paper, were calculated by means of the simple formula

$$M = \frac{RTC}{P},$$

where M = molecular weight; P = osmotic pressure; R = gas constant; C = concentration of protein in g./l. of solution; and T = absolute temperature. Except where explicitly

stated, the osmotic pressure experiments were carried out in a cold room at +2°.

The insulin used for all the investigations described in this paper was a sample of crystalline zinc insulin (Boots Pure Drug Co. Ltd.). Ultracentrifugal examination (Gutfreund & Ogston, 1946) showed that this material was homogeneous in solutions of pH 7·0–8·0. Electrophoretic analysis in the Tiselius apparatus, using the Philpot (1938) optical system for the observation of the moving boundaries, also confirmed the purity of the sample used.

Table 1. *Sedimentation constants of insulin*

Exp. no.	Insulin concentration (%)	pH	Molar composition of buffer solution: Citric acid	HCl	NaCl	Na_2HPO_4	S_{20} (corr.) $\times 10^{13}$
1*	0·4	3·4	0·055	—	0·1	0·045	3·10
2	0·6	3·0	0·079	—	0·2	0·041	3·36
3	0·15	2·5	0·091	—	0·2	0·017	—
4	0·75	2·2	0·098	—	0·2	0·004	2·59
5	1·20	1·0	—	0·1	0·2	—	2·78
6†	0·84	8·0	—	—	0·1	0·0095 + 0·0005 KH_2PO_4	3·47

* Quoted from Gutfreund & Ogston (1946).
† Solution used in Exp. 5 adjusted to pH 8 and dialyzed against phosphate buffer (see text).

Two types of buffer systems were employed as solvent: for solutions of pH 2·2–5·0, McIlvaine's (1921) citric acid-phosphate buffer; and for those of pH 5·0–8·8, mixtures of Na_2HPO_4 and KH_2PO_4 as described by Clark (1928). Solutions of pH 1 were prepared in 0·1 M-HCl.

After the protein solutions had been dialyzed against the appropriate buffer, the pH of the dialysate was determined with a glass electrode.

RESULTS

Gutfreund & Ogston (1946) found that insulin was polydisperse in solution of pH 3·4 and the first series of experiments described here was carried out to extend the study on the properties of more acidic insulin solutions. In Table 1 the sedimentation constants calculated from measurements on solutions of pH 3·4, 3·0, 2·2 and 1·0 respectively, are recorded together with details about composition of the buffer used as solvent and the protein concentration. Specimen photographs of the boundaries are shown in Fig. 1.

It can be seen from the width of the sedimenting boundaries shown in the sedimentation diagrams 1, 2, 3 and 4 that insulin in acid solutions is heterogeneous with respect to particle size. It can be presumed that these solutions contain insulin in various states of aggregation or dissociation. The spread of the heterogeneous boundaries decreases the accuracy of the values for S_{20}, particularly for the more dilute solutions. This, as well as dissociation due to dilution, may account for the difference in the value for S_{20} obtained from the ultracentrifuge Exps. 1 and 2. From Exp. 4 a lower sedimentation constant was calculated for the main boundary and the presence of a considerable amount of very slowly sedimenting material is evident from the boundary diagram. In a solution of pH 1·0 (Exp. 5) the sedimentation constant was somewhat higher than in a solution of pH 2·2. Furthermore, the boundary in the sedimentation diagram for Exp. 5 has a much more homogeneous appearance than that derived from all the other experiments on insulin in acid solutions.

The combined effect of dilution and acidity on insulin was also investigated. A solution containing 0·15 % insulin in buffer of pH 2·5 was examined in the ultracentrifuge (Exp. 3) and the sedimentation diagram shows clearly a marked increase in the proportion of very slowly sedimenting material compared with the more concentrated solution of Exp. 4.

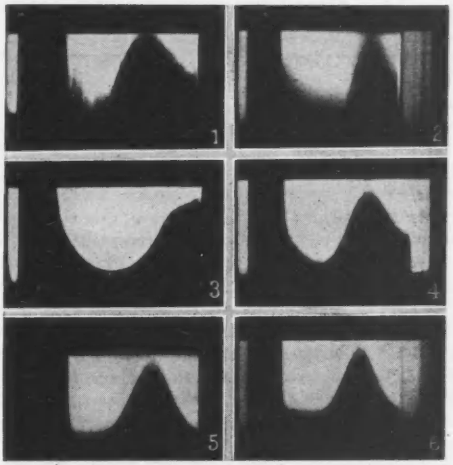

Fig. 1. Sedimentation diagrams for Exps. 1–6 numbered as in Table 1. The time (in min.) after reaching full speed was 80, 57, 80, 80, 60, 60, for diagrams 1–6 respectively.

As no clear boundary was formed the sedimentation constant could not be computed from Exp. 3.

The solution of pH 1·0, which had been used for sedimentation Exp. 5, was afterwards adjusted to pH 8·0, and dialyzed against phosphate buffer of that pH and again examined in the ultracentrifuge (Exp. 6); the sedimentation constant of insulin in this solution, $S_{20} \times 10^{13}$, was 3·47, which agrees within the experimental error with the value recorded for the sedimentation constant of unchanged insulin.

In the previous paper on the osmotic pressure of insulin solutions (Gutfreund, 1948) experiments were described in which phosphate buffer of pH 7·0 was used as solvent. It was necessary to dilute the buffer to 0·01 M to increase the solubility of insulin. In such dilute buffer a quite appreciable partial pressure due to excess ion activity was observed, and the necessary corrections were calculated from the results of membrane-potential measurements. In this paper the extrapolation procedure suggested by Adair & Robinson (1930) is used to interpret the results from measurements on solutions of insulin in 0·01 M-phosphate buffer of pH 7·5. The graph in Fig. 2 shows P/C plotted against C and gives several

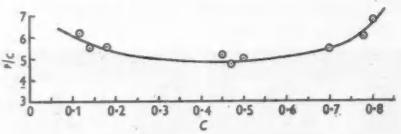

Fig. 2. Plot of P/C against C from osmotic-pressure measurements on solutions of insulin in 0·01 M-phosphate buffer of pH 7·5. P = osmotic pressure; C = concentration of protein in g./100 ml. solution.

interesting results. At the centre of the curve (C equal to 0·4–0·5 %) $P/C = 4·9$, corresponding to a molecular weight of 47,400, which is in good agreement with the value previously obtained at pH 7·0 and similar protein concentration (0·4–0·8 %). The value for P/C increases however both for $C > 0·5$ and $C < 0·3$. This is due to the membrane-potential effect in the more concentrated solutions and to the effect of dissociation, which has previously been described (Gutfreund, 1948) in the very dilute solutions.

Between pH 4·0–7·0 insulin is so insoluble as to make any investigations on solutions very difficult. Between pH 7·0 and 7·5 solutions up to nearly 1 % could be obtained in very dilute buffer. At pH lower than 4·0 and higher than 7·5 it was possible to obtain insulin solutions, containing up to 1 % protein, in the presence of sufficient salt to eliminate the partial pressure due to excess ion activity. I have therefore made all the buffer solutions of those outside pH regions, 0·2 M with respect to NaCl. The following series of experiments was carried out at pH 1 (0·1 M-HCl, 0·2 M-NaCl) to find out whether P/C remained constant over a range of concentrations 0·5–1·0 % of protein, under those conditions.

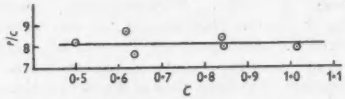

Fig. 3. Plot of P/C against C from osmotic-pressure measurements on solutions of insulin in 0·1 M-HCl, 0·2 M-NaCl. P = osmotic pressure; C = concentration of protein in g./100 ml. solution.

Fig. 3 is a graphical representation of the measurements on solutions of pH 1. The molecular weight, calculated from the results of the six experiments recorded here, is 28,500 ± 1100 (standard deviation from the mean). The ratio P/C is constant over the range of C investigated, and it can therefore be concluded that the low molecular weight observed is due to dissociation in acid solutions and not to the effect of the electric charge of the protein molecules. These experiments also demonstrate the accuracy to be expected from osmotic-pressure measurements on solutions of similar concentration.

A series of measurements of the osmotic pressure of insulin solutions (protein concentration 0·6–0·9 %) over a range of pH from 1·0 to 8·8 was then carried out, and the molecular weight values, recorded in Table 2, are in each case the means of the results calculated from several osmotic-pressure measurements.

Table 2. *The dependence of the molecular weight of insulin, in solutions of protein concentration* 0·6–0·9 %, *upon the* pH *of the solution*

pH	Molecular weight calculated from osmotic-pressure measurements
1·0	28,500
2·2	22,000
2·5	22,000
3·0	22,000
7·0	47,700
7·5	47,400
8·0	40,000
8·8	28,000

It is clearly indicated by these observations that insulin dissociates into subunits, both in acid and alkaline solutions. The reversibility of this dissociation was examined by adjusting both acid and alkaline solutions of insulin to pH 7·5 and it was found that re-association to a molecular weight of 47,000–48,000 occurred.

The observed re-aggregation of the insulin subunits between pH 2·2 and 1·0 suggested that it might be possible to study the fibrous polymerization in acid solutions described by Waugh (1946). However, on changing the concentration of HCl

from 0·1 to 1·0M most of the insulin in solution was precipitated and no measurements were made on more acidic solutions.

An observation, indicating the presence simultaneously of insulin in different states of aggregation, was then made. A buffer solution of pH 8·0 was saturated with insulin at a temperature of 30°. This solution, containing after centrifugation 1·1% insulin, was stored in a cold room over night and a crop of crystals was formed. Though the mean molecular weight of insulin in this solution was 40,000, the crystals which deposited were found to be isomorphous with those described by Crowfoot (1938).

From the results of the experiments so far described it is clear that in acid solutions the pH of maximum dissociation is between 2 and 3. The results of the ultracentrifuge Exps. 3 and 4 show that the degree of dissociation is dependent upon insulin concentration. The combined effect of pH and dilution was studied by a series of osmotic-pressure measurements on solutions of pH 2·5 containing 0·04–2·4% of insulin. The results of these experiments are recorded in Fig. 4 where the molecular

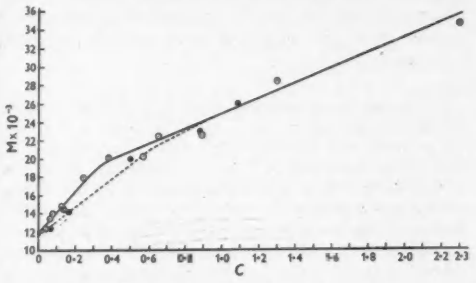

Fig. 4. Plot of molecular weight of insulin against concentration calculated from osmotic-pressure measurements in citric acid-phosphate buffer (pH 2·5). Empty circles represent measurements at 2°, full circles represent measurements at 23°.

weight is plotted against insulin concentration. Two curves are shown: one represents the results of measurements at 2°, the other those of measurements at 23°. Both curves can be extrapolated to a value of 12,000 ± 500 (standard deviation of the individual observations) for the molecular weight at zero concentration. A consideration of the accuracy of the measurements is particularly important for very dilute solutions (0·04–0·1% of protein). The accuracy of the molecular weight calculated from an osmotic-pressure measurement depends upon the accuracy of the estimation of the protein concentration and pressure. For the smallest values of concentration and pressure, measured for the calculation of the data in Fig. 4, the accuracy was ± 5% in each case.

DISCUSSION

From the results reported in this paper and from the data given by Gutfreund (1948) it can be concluded that the maximum molecular weight of insulin is 48,000. This value is found for neutral solutions (pH 7·0–7·5) of protein concentration 0·4–0·9%. Under these special conditions insulin is homogeneous with respect to particle size. In the previous paper it has been shown that insulin of molecular weight 48,000 is not very stable, as dissociation into smaller units occurs on dilution to protein concentrations of less than 0·3%.

The investigation of the effect of pH on the molecular weight of insulin showed that dissociation of insulin occurred both in acid and alkaline solutions. In acid solutions the optimum pH for dissociation is pH 2·0–3·0. pH 1 is the critical pH for the beginning of the re-aggregation resulting in more acidic solutions, in the formation of a fibrous precipitate. The results of ultracentrifugal and osmotic-pressure measurements are in agreement about the dissociation in acid solutions and the beginning of re-aggregation in solutions of pH 1. When establishment of equilibrium between dissociated and associated insulin molecules is not slower than the resolution produced by sedimentation, one can expect to get a spreading boundary giving a mean sedimentation constant, rather than two or more separate component boundaries. It is at present difficult to give a definite explanation for the results obtained by Gutfreund & Ogston (1946) on ultracentrifugal examination of insulin in alkaline solutions. They observed no change in the sedimentation constant nor any spreading of the boundary. However, as stated on p. 544 of this paper, some slowly-sedimenting material could be seen in the sedimentation diagrams from experiments on solutions more alkaline than pH 8·0. It is possible that the difference in the appearance of sedimenting boundaries in acid and alkaline solutions is due to a difference in the speed of the dissociation equilibrium under the two respective conditions. A very slow equilibrium may account for the type of boundaries obtained in alkaline solutions.

The last series of osmotic-pressure measurements described in this paper was undertaken to investigate the size of the units into which insulin dissociates. Though the results indicate that the molecular weight of insulin in infinitely dilute solution is 12,000, there is no direct proof from these data as to the homogeneity of the dissociation products. In spite of variations of temperature, pH and concentration, the molecular weight was never found to be below 12,000 and the two curves from measurements at 2° and 23° converge at the point of molecular weight 12,000 (Fig. 4); this can be assumed to be the molecular weight of the dissociation product.

The results of the crystallographic investigations (Crowfoot, 1938) and of the chemical analysis (Chibnall, 1946) do not by themselves prove the existence of a subunit of molecular weight 12,000, but they are consistent with that hypothesis. The unit cell in crystalline insulin is composed of three identical parts and the unit-cell weight is 36,000 (recalculated for a moisture content of 10–11% for insulin crystals). The minimum molecular weight calculated from amino-acid analysis is 12,100 with a standard deviation of 530, from the values quoted by Chibnall (1946).

The reversibility of the dissociation reaction of insulin was demonstrated by the ultracentrifugal Exps. 5 and 6, by osmotic-pressure measurements and by the work of Waugh (1946). Gutfreund (1948) calculated the total heat change occurring on dissociation of insulin molecules of molecular weight 48,000 into four identical subunits. At pH 7·0, ΔH was found to be 29,000 g. calories (for 1 mol. insulin). Similar calculations were not done for the data recorded in Fig. 4, as a series of measurements at each temperature for one point of the concentration scale has to be made to get results of sufficient accuracy for subsequent calculation of the relative concentrations of associated and dissociated insulin. The data so far obtained are, however, quite accurate enough to show the relation between concentration and molecular weight.

Since the degree of dissociation of insulin molecules is dependent upon pH, temperature and concentration, it can be concluded that the forces holding the subunits together involve the ionizing groups of the protein. It is very difficult to differentiate between electrostatic forces and hydrogen bonds as the latter are often affected by the ionization of certain groups. A study of the influence of the ionic strength of the solvent buffer upon dissociation, which is now being pursued, may give further information on that point.

SUMMARY

1. Insulin has a maximum molecular weight (47,000–48,000) in solutions of pH 7·0–7·5 at protein concentrations of 0·4–0·9%. Under those conditions it is homogeneous with respect to particle size.

2. Molecules of this weight dissociate reversibly on change of pH to above 7·5 or below 4, as well as on dilution. The optimum pH for dissociation was found to be 2·0–3·0.

3. On studying the combined effect of pH, dilution and temperature upon dissociation it was found that the minimum molecular weight was $12,000 \pm 500$. Other evidence suggesting that insulin molecules in solution were composed of subunits of molecular weight 12,000 is summarized.

4. It is suggested that units of molecular weight 12,000 are bound by weak electrostatic forces.

5. The dissociation phenomena have been studied by osmotic pressure and by ultracentrifugal measurements. The two methods gave results in good agreement.

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REFERENCES

Adair, G. S. (1925). *Proc. Roy. Soc.* A, **108**, 627.
Adair, G. S. & Robinson, M. E. (1930). *Biochem. J.* **24**, 1864.
Chibnall, A. C. (1946). *J. int. Soc. Leath. Chem.* **30**, 1.
Clark, W. M. (1928). *The Determination of Hydrogen Ions.* London: Baillière, Tindall & Cox.
Crowfoot, D. (1938). *Proc. Roy. Soc.* A, **164**, 580.
Gutfreund, H. (1948). *Biochem. J.* **42**, 156.
Gutfreund, H. & Ogston, A. G. (1946). *Biochem. J.* **40**, 432.
McIlvaine, T. C. (1921). *J. biol. Chem.* **49**, 183.
Philpot, J. St L. (1938). *Nature, Lond.*, **141**, 283.
Sjögren, B. & Svedberg, T. (1931). *J. Amer. chem. Soc.* **53**, 2657.
Waugh, D. J. (1944). *J. Amer. chem. Soc.* **66**, 663.
Waugh, D. J. (1945). *Fed. Proc.* **4**, 1.
Waugh, D. J. (1946). *J. Amer. chem. Soc.* **68**, 247.

The Distribution of Acid-soluble Phosphates in the Fatty Liver

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Although increased attention has recently been paid to the function of the acid-soluble phosphates in liver metabolism, the bulk of the investigations has been concerned with changes in the distribution of organic phosphates brought about by variations in diet (Flock, Bollman & Mann, 1936*a*; Kaplan & Greenberg, 1944*b*; Rapoport, Leva & Guest, 1943 *a*, *b*). The effect of injections of glucose (Kaplan & Greenberg, 1944*a*), insulin (Cori & Goltz, 1925; Nelson, Rapoport, Guest & Mirsky, 1942; Kaplan & Greenberg, 1943, 1944*a*) and various enzyme inhibitors has also been studied. Little work, however, appears to have been done on the acid-soluble phosphates of fatty livers, although Flock *et al.* (1936*a*), working with dogs maintained on a high fat diet and thus having fatty livers, have stated that if the diet is given for 3 weeks or longer there is a decrease of inorganic phosphate to 60% of the normal value. The same authors (Flock *et al.* 1936*b*) also subjected dogs to carbon tetrachloride anaesthesia for 1 hr. on four successive days, but found that the distribution of the various acid-soluble-phosphate fractions from the livers was unaffected. The distribution of acid-soluble phosphates from fatty livers has recently become of added interest in view of the importance of adenylic acid in the oxidation of butyrate (Munoz & Leloir, 1943).

It has already been shown that the oxygen consumption and the acetoacetic acid production of liver slices from guinea pigs treated with carbon tetrachloride is considerably in excess of normal (Ennor, 1942). This together with the fact that adenosine triphosphate (*ATP*) is essential for the oxidation of fatty acids (Lehninger, 1945 *a*, *b*) suggested the likelihood of a correlation between the adenosine polyphosphate (*APP*) content and the increased metabolism of these fatty livers. Experiments designed to test this possibility have been carried out and are described in this communication. Preliminary results were reported earlier (Ennor & Stocken, 1947).

EXPERIMENTAL

Expression of results

All the above workers have expressed their results on the basis of wet weight of tissue, but, since it has been shown that the liver is subject to wide variations in water, fat and glycogen content (cf. Higgins, Berkson & Flock, 1932, 1933), it is clear that reference to a weight which includes these substances is open to criticism. Flock *et al.* (1936*a*) commented on this, but were unable to show that calculation on the basis of dry, fat-free weight altered their conclusions. Since variations in the acid-soluble organic phosphates of liver reflect variations in the metabolism of carbohydrate, protein and fat, and thus in the activities of the various enzymes concerned, it seems desirable to express all results in a more satisfactory manner. Expression on the basis of percentage N or nucleoprotein N would largely overcome these objections, and the figures so obtained could be more readily capable of interpretation from the standpoint of metabolic activity. Nucleoprotein N would appear to be the choice, but, because of the technical difficulties associated with its estimation when a large number of other determinations have to be done, all the results quoted in this communication have been expressed on the basis of total liver N. However, for the purpose of comparison with the results of other workers, some of our results are also quoted on the basis of wet liver weight. It is realized that the presence of labile protein in the liver will perhaps complicate the picture, but it is thought that the error introduced will be small, particularly as the mobile protein stores are largely depleted following a 24 hr. fast.

Methods

Phosphorus estimation. Inorganic phosphate was determined by the method of Berenblum & Chain (1938) and total P by the same method after ashing with HNO_3 and H_2SO_4.

Nitrogen. Duplicate samples (*c.* 0·1 g.) of unfrozen liver were ashed with the digestion mixture described by Campbell & Hanna (1937), and after suitable dilution samples were distilled in the apparatus of Markham (1942). The distillate was collected in a boric acid buffer used by Conway (1942) and titrated with 0·01 N-H_2SO_4.

Fat. In this paper 'fat' refers to the material extractable from the liver by means of a 70/30 (v/v) ethanol-diethyl ether mixture.

Creatine. The method used was that of Eggleton, Elsden & Gough (1943).

Pentose. This was estimated according to the method of Meijbaum (1939), but as suggested by Stone (1943) a solution of barium adenosine triphosphate was used as a reference standard.

Treatment of animals. Male guinea pigs, of *c.* 300 g. body weight, fed on a liberal diet of bran, cabbage and swede, were used throughout. Treated animals were given, on successive days, three subcutaneous injections each of 0·2–0·3 ml. of a mixture containing equal volumes of carbon tetrachloride and arachis oil. Free access was allowed to water, but food was withheld from both treated and control animals for 24 hr. prior to the experiment. Treated animals were killed 24 or 48 hr. after the final injection.

Preparation of extracts. The animals were stunned by a blow on the head and the neck vessels severed. The liver was rapidly removed and the major portion dropped into liquid air; the remainder was analyzed for N, total P and fat. After a preliminary grinding in a mortar chilled with liquid air, duplicate samples of 2–3 g. of the frozen liver were homogenized with 4 ml. of ice-cold 10% (w/v) trichloroacetic acid (*TCA*). The homogenizer was similar to that described by Potter & Elvehjem (1936) but was provided with a stainless-steel pestle. The homogenate was diluted with 20 ml. of 5% (w/v) *TCA* and, after centrifugation, the residue was re-extracted with a further 20 ml. of the 5% acid. Since this procedure served to extract not less than 97% of the acid-soluble phosphates, further extraction was considered unnecessary. The combined extracts were made up to a total volume of 50 ml. with 5% *TCA*, filtered if necessary, and analyzed for inorganic and total P.

RESULTS

There is a marked difference in the total-P contents of the fresh livers from the treated and the control animals (Table 1). A similar difference is also apparent when the total- and organic-P contents of the *TCA* extracts are considered. Thus, when the figures are expressed on the basis of the wet weight of the liver, the values for the total P and total and organic acid-soluble P are higher in the control group than in the treated. Expression on the basis of the N content of the liver, however, leads to a reversal of this result.

Because of the facts that *ATP* is essential for the oxidation of fat, that there is a well recognized association between oxidation and phosphorylation, and that there is an increase in the rate of O_2 uptake in fatty livers (cf. Meier & Thoenes, 1933; Ennor, 1942), it might be anticipated that there would be an increase in the high energy phosphate levels in such livers. Attention was, therefore, in the first instance, focused on the compounds hydrolyzable in 7 min. in N-acid at 100°. A separation of *APP* from other easily hydrolyzable compounds, such as acetyl phosphate and phosphocreatine, and from many organic phosphates, is possible by barium precipitation and this was carried out on the *TCA* extracts. Several methods have been described for the preliminary separation, but that described by Le Page & Umbreit (1945) is the most convenient. This procedure has the advantage over the alternative one, in which solid baryta is used, in that it is much quicker, but it should be noted that the use of NaOH for the neutralization of *TCA* extracts results in the incomplete precipitation of inorganic phosphate at pH 8·2. Kosterlitz & Ritchie (1943) have commented on this, although it is not clear what concentration of Ba^{++} was present in their extracts. Under our conditions adjustment to pH 9·5, as recommended by these workers, results in a decrease of the solubility of barium phosphate to less than 1 μg. of P/ml. as compared with approximately 2·5 μg. of P/ml. at pH 8·2. In the bulk of our experiments, however, this latter pH has been chosen since the faint pink end point of phenolphthalein is easily reproducible. Further, the small amounts of inorganic phosphate which appear in the mother liquor do not introduce any complication.

Table 1. *Nitrogen, phosphorus and fat analyses of livers and liver extracts from control guinea pigs and from guinea pigs treated with carbon tetrachloride*

Fresh liver				*TCA* extract			
		Total P		Total P		Organic P	
Fat (%)	N (%)	(mg./g. N)	(%)	(mg./g. N)	(mg./100 g.)	(mg./g. N)	(mg./100 g.)
				Control animals			
—	3·51	94	0·33	24·7	86·6	17·9	62·6
6·6	3·58	91	0·33	26·5	95·0	20·6	73·9
6·6	3·73	92	0·34	27·4	102·0	21·7	80·7
—	3·54	98	0·35	29·2	102·0	22·8	79·4
7·8	3·63	95	0·34	27·4	99·4	20·6	74·8
6·0	3·06	—	—	27·6	84·6	20·4	62·4
—	3·78	93	0·35	28·3	107·0	22·5	85·4
5·4	3·65	—	—	25·5	93·0	19·1	69·6
9·0	3·57	—	—	27·0	96·5	20·3	72·6
	Mean 3·56	**94**	**0·34**	**27·1**	**96·2**	**20·7**	**73·5**
				Animals treated with carbon tetrachloride			
16·2	1·84	122	0·23	36·8	67·8	25·4	47·0
12·5	2·20	134	0·29	30·4	66·9	21·9	48·2
10·6	2·46	100	0·24	33·0	82·0	24·4	60·5
13·0	2·30	107	0·24	36·1	83·0	29·0	66·7
14·6	2·20	—	—	35·1	77·3	27·4	60·4
14·5	1·95	—	—	39·8	77·6	31·9	62·2
	Mean 2·16	**115**	**0·25**	**35·2**	**75·6**	**26·7**	**57·5**

Stone (1943) has found that the amount of *ATP* which is precipitable from *TCA* extracts of brain tissue depends on the concentration of $Ca(OH)_2$. Experiments to determine the amount of *ATP* precipitable by barium under our conditions indicated that there was no significant difference in the amounts precipitated when the concentration of Ba^{++} ranged from M/8 to M/30 and that the amount of *ATP* which remained in the mother liquor was less than 0·2 μg. P/ml. The final procedure adopted for the separation was, therefore, as follows.

To 30 ml. of the *TCA* extract was added 1 ml. of 20% (w/v) $BaCl_2$ and sufficient 5 N-NaOH to bring the pH to 8·2 with phenolphthalein as internal indicator. The solution was allowed to stand for 20 min. to ensure complete flocculation and then centrifuged. The supernatant fluid was made up to 50 ml.: the precipitate was dissolved in the minimum volume of 0·1 N-HCl and made up to 25 ml. with water. All manipulations were carried out at 0°. Except where stated the barium was not removed from the various fractions prepared from the *TCA* extracts, since $BaSO_4$ does not interfere with the method used for the estimation of P.

It is clear that there is a marked increase in the 7 min.-hydrolyzable compounds present in the barium-insoluble fraction of *TCA* extracts of the fatty liver. Moreover, this increase is demonstrable whether expressed on the basis of wet weight or N, and in general may be correlated with the fat content (Table 2).

analytical methods have been criticized by Kalckar (1945), although it is not clear just how his criticism can be applied to the results obtained by these workers. A more satisfactory method for the identification of the compound responsible for the liberation of the phosphate under the conditions of hydrolysis lies in the determination of the N, P and pentose ratio. By this method Le Page & Umbreit (1945) and Le Page (1946) found that in the livers of rats the ratio of *ADP* to *ATP* is about 17 : 1. On the other hand Rapoport (1945), using myosin and a specific nucleotidase, produced evidence indicating a ratio of 2 : 1 in the fed, and 5 : 1 in the starved, liver.

We have also determined the ratios of N : 7 min.-labile P : pentose in the reprecipitated barium insoluble fractions. The normal liver gave a ratio of 5·0 : 1·3 : 0·9 and the fatty liver 5·0 : 1·3 : 1·0, and since *ADP* requires a ratio of 5·0 : 1·0 : 1·0 it might be concluded that a considerable proportion of the P released is derived from *ADP*. This conclusion, however, was not confirmed when the adenosine triphosphatase (*ATP*-ase) method (Rapoport & Nelson, 1945) for the determination of *ATP* was employed. From the N, P and pentose determinations a molar ratio of *ATP* to *ADP* ranging from 1 : 1 to 1 : 7 was indicated, whereas the ratios as determined by the myosin *ATP*-ase ranged from > 90 : 1 to 2 : 1. Because of the high degree of substrate specificity of the *ATP*-ase it would seem that

Table 2. *Amounts of phosphorus released in 7 min.* (P_7) *at 100° in* N-HCl *from the barium-insoluble fractions of TCA extracts prepared from livers of control guinea pigs and of guinea pigs treated with carbon tetrachloride*

Control animals				Animals treated with carbon tetrachloride			
Fat (%)	N (%)	P_7 (mg./100 g.)	P_7 (mg./g. N)	Fat (%)	N (%)	P_7 (mg./100 g.)	P_7 (mg./g. N)
—	3·89	4·8	1·2	23·0	2·15	9·8	4·5
—	3·58	6·4	1·7	10·8	2·53	5·5	2·1
—	3·63	3·9	1·1	15·9	1·86	6·9	3·7
—	3·25	3·3	1·0	13·0	1·77	5·3	3·0
—	3·57	3·8	1·1	16·2	1·84	6·1	3·3
9·8	3·48	4·7	1·4	12·5	2·20	6·2	2·8
—	3·51	5·7	1·6	13·4	2·79	5·9	2·1
6·6	3·58	1·9	0·6	14·5	1·95	8·8	4·5
6·6	3·73	4·5	1·1	10·6	2·46	6·9	2·9
6·0	3·06	6·3	2·0	13·0	2·30	8·0	3·6
5·4	3·65	7·4	2·0	14·6	2·20	8·5	3·9
9·0	3·57	7·5	2·1	—	—	—	—
8·8	3·93	8·3	2·1	—	—	—	—
		Mean 5·3	1·4			7·1	3·3

Most workers have been unconcerned with the origin of the inorganic phosphate released from this fraction in 7 min. at 100° in N-acid and many have, in fact, referred to it as *ATP*. In muscle extracts this phosphate certainly arises from *ATP*, but the picture in liver is somewhat different, as Kalckar (1945) has pointed out. Kaplan & Greenberg (1944*c*) concluded that the compound is *ATP*, but their

these figures are the more reliable. The explanation of these differences lies in the possibility of contamination of the fraction with some N compound, the presence of which will greatly affect the ratio of labile P to N.

Having established that there was a marked increase in the 7 min.-hydrolyzable fraction, it was considered desirable to investigate the possibility

of changes in the concentration of compounds hydrolyzable at longer intervals. Hydrolysis curves were determined on samples of both the original *TCA* extracts and the barium-insoluble fraction. All hydrolyses were carried out in N-HCl at 100° for periods of 7, 15, 30, 60 and 180 min., and on removal from the bath the tubes were rapidly cooled in a freezing mixture and then analyzed for the inorganic phosphate released. Figs. 1 and 2 illustrate curves

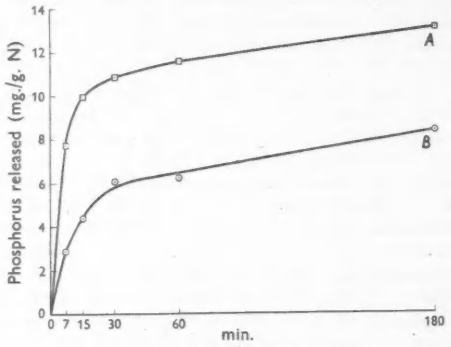

Fig. 1. Hydrolysis curves illustrating the amounts of inorganic P released at 100° in N-HCl for varying periods from *TCA* extracts of a fatty liver (*A*) and a normal liver (*B*). All points on the curves represent the means of at least two determinations.

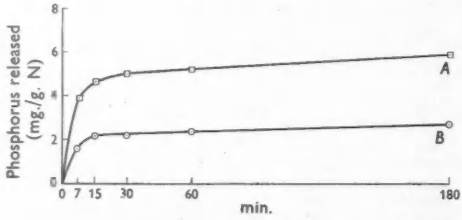

Fig. 2. Hydrolysis curves illustrating the amounts of inorganic P released at 100° in N-HCl for varying periods from the barium-precipitable fraction of *TCA* extracts, the hydrolysis curves of which are shown in Fig. 1. *A*, fatty liver, *B*, normal liver. All points represent the means of at least two determinations.

typical of those obtained from normal and fatty livers. It will be seen that the general shape of the curves is similar, but those illustrating the behaviour of the phosphate compounds in the fatty liver are on a higher level, due largely to the presence of increased amounts of compounds hydrolyzable in 7 min. This is more clearly seen from the data given in Table 3. At the 7–15 min. interval in the *TCA* extracts the difference in favour of the fatty liver is proportionally the same as in the 0–7 min. interval. For the remaining intervals no certain conclusions can be reached as to any differences between the normal and fatty liver.

The compounds which appear in the *TCA* extracts of liver include *ATP*, *ADP*, glucose-1-phosphate, phosphocreatine, phosphopyruvic acid, hexosediphosphate, fructose-6-phosphate, phosphoglyceric acid and possibly acetyl phosphate. Breakdown of these compounds, if present in our extracts, must therefore be responsible for the differences between the normal and the fatty liver in the 0–7 min. period. Since the first four compounds and acetyl phosphate are completely hydrolyzed in this interval, they are likely to be responsible for by far the greater part of the P released.

ATP, *ADP* and hexosediphosphate are precipitable by barium and it follows that the differences between the amounts of P released in 7 min. in the *TCA* extracts and in the barium-insoluble fraction will show whether the increased amount of *APP* is, in fact, entirely responsible for the difference noted in the 7 min.-hydrolyzable compounds present in the *TCA* extract. These differences, together with those at other intervals, are presented in Table 4, and in most instances were obtained by direct estimation in the barium-soluble fraction and not from the difference between the values for the *TCA* extracts and the barium-insoluble fraction.

These figures also show a marked difference between the normal and fatty livers, and it is clear that the increased amount of P released in 7 min. from *TCA* extracts of fatty livers (Table 2) is not entirely due to the differences which have been noted in the barium-precipitable fraction. Thus, in addition to a rise in the *APP* content, there is also an increase in readily hydrolyzable compounds in the barium soluble fraction. These differences persist to about the same degree over the 7–15 min. interval, whilst the amounts released over the 15–30 min. period are of the same order. Rather more P is apparently released between 30 and 60 min. with the fatty livers, but here, as in the next interval, the results are too variable to permit any definite conclusions. No attempt has been made, as yet, to identify the compounds responsible for any differences noted at the longer time intervals since interest was, in the main, centred on the more readily hydrolyzable compounds.

Most workers, who have been concerned with the estimation and the identification of labile P compounds, appear to have used either the method of Fiske & Subbarow (1925) or modifications thereof. In these methods the phosphomolybdate is reduced in aqueous solution and it is essential to adhere strictly to carefully standardized conditions in order to achieve consistent and dependable results (cf. Stone, 1943).

Table 3. *Amounts of phosphorus released at various time intervals in* N-HCl *at* 100° *from TCA extracts and barium-insoluble fractions prepared from livers of control guinea pigs and of guinea pigs treated with carbon tetrachloride*

TCA EXTRACT

0–7 min.		7–15 min.		15–30 min.		30–60 min.		60–180 min.		Total, 180 min.	
(mg./ g. N)	(mg./ 100 g.)	(mg./ g. N)	(mg./ 100 g.)	(mg./ g. N)	(mg./ 100 g.)	(mg./ g. N)	(mg./ 100 g.)	(mg./ g. N)	(mg./ 100 g.)	(mg./ g. N)	(mg./ 100 g.)
Animals treated with carbon tetrachloride											
7·8	17·9	—	—	—	—	—	—	—	—	—	—
6·2	14·7	—	—	—	—	—	—	—	—	—	—
6·3	11·6	2·8	5·3	1·4	2·6	1·0	1·9	0·8	1·4	13·1	24·2
5·5	11·9	2·4	5·3	1·0	2·3	0·7	1·6	1·5	3·7	10·6	23·4
5·0	13·2	2·1	5·8	0·5	1·4	0·9	2·7	—	—	—	—
9·6	18·6	3·2	5·6	1·1	2·2	1·0	1·7	1·7	3·5	15·3	29·8
7·8	17·2	2·2	4·7	0·9	1·9	0·7	1·6	1·5	3·5	14·3	31·5
Mean 6·9	**15·0**	**2·5**	**5·3**	**1·0**	**2·1**	**0·9**	**1·9**	**1·4**	**3·0**	**13·3**	**27·2**
Control animals											
2·9	10·4	1·5	5·4	1·7	5·8	0·0	0·0	2·2	8·0	9·5	35·3
3·9	14·3	1·1	4·1	1·0	3·6	0·1	0·2	1·9	7·0	11·1	31·4
4·2	14·9	1·4	5·0	0·5	1·8	0·0	0·0	2·2	7·8	12·0	43·1
4·0	15·8	1·2	4·9	0·8	3·0	1·2	4·6	1·1	4·7	10·0	39·4
2·4	8·6	—	—	—	—	—	—	—	—	—	—
3·0	11·8	—	—	—	—	—	—	—	—	—	—
4·0	14·3	—	—	—	—	—	—	—	—	—	—
3·0	11·0	—	—	—	—	—	—	—	—	—	—
4·5	15·7	1·6	5·6	0·3	1·3	1·2	5·2	—	—	—	—
Mean 3·5	**13·0**	**1·4**	**5·0**	—	—	—	—	—	—	—	—

BARIUM-INSOLUBLE FRACTION

Animals treated with carbon tetrachloride											
2·6	8·0	—	—	—	—	—	—	—	—	—	—
2·9	6·9	—	—	—	—	—	—	—	—	—	—
3·3	6·1	0·6	1·4	0·6	1·0	0·2	0·3	0·5	1·0	3·1	5·6
2·8	6·2	0·7	1·5	0·2	0·3	0·2	0·6	0·5	0·8	2·9	6·6
2·1	5·9	0·8	2·4	0·4	1·0	0·0	0·0	—	—	—	—
4·5	8·8	1·4	2·6	0·5	1·0	0·5	0·9	1·0	2·0	1·6	3·2
3·9	8·5	0·7	1·7	0·4	0·8	0·2	0·3	0·7	1·6	3·0	6·6
Mean 3·2	**7·2**	**0·8**	**1·9**	—	—	—	—	—	—	—	—
Control animals											
1·6	5·6	0·6	1·9	0·0	0·0	0·1	0·3	0·4	1·4	1·8	6·3
2·0	7·4	0·5	1·9	0·4	1·6	0·0	0·0	0·9	3·3	1·8	6·4
2·1	7·5	0·6	2·0	0·3	1·1	0·0	0·0	0·4	1·4	2·8	9·8
2·1	8·3	0·6	2·3	0·1	0·2	0·7	2·6	0·2	0·8	1·8	7·1
Mean 1·9	**7·2**	**0·6**	**2·0**	—	—	—	—	—	—	—	—

The presence of phosphocreatine in rat-liver extracts has been denied (Flock *et al.* 1936*a*) and affirmed (Le Page & Umbreit, 1945; Le Page, 1946), although in no case have any specific methods for its identification been employed. The failure of Flock *et al.* (1936*a*) to detect phosphocreatine may have been due to their use of mercury for the removal of glycogen from the extracts. This metal is known to produce rapid catalytic decomposition of phosphocreatine (Fiske & Subbarow, 1929). Since the latter is very labile in acid molybdate, it is customary to follow either the comparative colorimetric procedure employed by Fiske & Subbarow (1929), or to precipitate the inorganic phosphate and then obtain the phosphocreatine by difference. A somewhat similar procedure was adopted in the first instance for the determination of acetyl phosphate (Lipmann & Tuttle, 1944). In the method for the determination of P devised by Berenblum & Chain (1938), however, the acid molybdate-labile compounds, if present, are only in contact with the acid phase for 1 min. during the extraction of the phosphomolybdate complex by *iso*butanol. The aqueous layer is then discarded and it is thus only the P released in this short interval which adds to the 'true' inorganic phosphate present. According to Fiske & Subbarow (1929) 28·9 % of the phosphocreatine present is hydrolyzed in 3 min. at 18° in acid molybdate and Lipmann & Tuttle (1944) have shown that acetyl phosphate is 75 % decomposed in *c.* 2 min. at 23°.

Table 4. *Amounts of phosphorus (mg./g. N) released in the indicated intervals from compounds present in the barium-soluble fraction of TCA extracts of livers of control guinea pigs and of guinea pigs treated with carbon tetrachloride, and hydrolyzable in* N-HCl *at* 100°

0–7 min.	7–15 min.	15–30 min.	30–60 min.	60–180 min.	Total, 180 min.
Animals treated with carbon tetrachloride					
3·0	2·2	0·8	0·8	0·3	10·0
2·7	1·7	0·8	0·5	1·0	7·7
2·9	1·3	0·1	0·9	—	—
5·1	1·8	0·6	0·5	1·2	13·2
3·9	1·5	0·5	0·5	0·8	11·0
3·3	—	—	—	—	—
5·2	—	—	—	—	—
Mean 3·7	1·7	—	—	—	—
Control animals					
1·3	0·9	1·7	0·0	1·8	7·7
1·9	0·6	1·0	0·0	1·0	9·0
2·1	0·8	0·2	0·0	1·8	9·4
1·9	0·6	0·8	0·5	1·9	8·2
1·8	—	—	—	—	—
1·9	—	—	—	—	—
2·0	—	—	—	—	—
Mean 1·8	0·7	—	—	—	—

It is thus clear that these compounds will only be estimated in part as inorganic phosphate when the Berenblum & Chain (1938) method is employed, and the remainder would be estimated together with other labile compounds in the 7 min.-hydrolyzable fraction. The compounds which are likely to make a substantial contribution to the amount of P released in 7 min. in the barium-soluble fraction are phosphocreatine, glucose-1-phosphate, phosphopyruvic acid and possibly acetyl phosphate. It follows, therefore, that if these are present in increased amounts in the fatty liver, they would account in some measure for the increase in the 7 min.-hydrolyzable material seen in the barium-soluble salts derived from the *TCA* extracts of the fatty liver.

The presence of a molybdate-labile compound or compounds in the *TCA* extracts was indicated by increases in the amounts of P measurable after allowing the sample to remain in the acid molybdate for 30 min. at 30° before extraction of the phosphomolybdate complex with *iso*butanol. Under these conditions we have established that phosphocreatine is completely decomposed and that no P is split from *ADP* or *ATP*.

The results (Table 5) indicate that there exists a marked difference between the control and the treated series and that, moreover, the normal liver contains some acid molybdate-labile compound or compounds. Since the fatty livers from guinea pigs treated with carbon tetrachloride exhibit an increased acetoacetic acid production (Ennor, 1942), it may be presumed that increased amounts of fat are being oxidized. It would seem, therefore, that such livers should contain demonstrable amounts of acyl phosphates, if these are formed from the fatty acid chain as has been suggested by Lehninger (1945*b*). We have made repeated attempts to detect the presence of this compound by the hydroxylamine method described by Lipmann & Tuttle (1945). In no case has a positive reaction been obtained, even though extracts have been employed containing amounts of labile P corresponding to 60 μg. of acetyl phosphate/ml.

Table 5. *Acid molybdate-labile compounds present in TCA extracts of livers from control guinea pigs and from guinea pigs treated with carbon tetrachloride*

Control animals		Animals treated with carbon tetrachloride	
P (mg./g. N)	P (mg./100 g.)	P (mg./g. N)	P (mg./100 g.)
0·9	2·7	—	—
0·2	0·6	1·5	3·1
0·4	1·4	1·7	2·8
0·8	2·8	2·1	4·1
0·2	0·7	1·8	4·8
0·5	1·6	—	—
Mean 0·5	1·6	1·8	3·7

The possibility of the presence in the extracts of substances which might interfere with the reaction has been checked by the detection of added succinylhydroxamic acid corresponding to 25 μg. of acetyl phosphate.

It appears, therefore, that the suggestion of Kaplan & Greenberg (1944*c*) that the acid molybdate-labile compound present in their extracts was acetyl phosphate is unlikely. It may also be noted that Kaplan & Greenberg based their suggestion upon the premise that phosphocreatine was not

present in the liver. This is in disagreement with the results of Le Page & Umbreit (1945), although these latter workers did not provide unequivocal proof that the labile compound was phosphocreatine. We have tried to obtain more certain evidence by correlating the labile P with the bound creatine. Such a correlation has been achieved with extracts from both normal and fatty livers, but because of the presence of inhibitors which interfere with the estimation of free creatine, the analytical methods are being investigated. It is hoped that the results will be published later. For the moment, however, from these unpublished experiments, it is considered that the acid molybdate-labile P arises for the most part from phosphocreatine, and certainly not from acetyl phosphate.

DISCUSSION

Since the technique adopted throughout these investigations differs, in some respects, from that of other workers it is desirable to point out these differences and to discuss any bearing they may have on the results. It is generally accepted that true, normal 'resting values' for lactic acid and *ATP* in brain and muscle will not be obtained unless the organs are removed under anaesthesia. We have not used any anaesthetic before removal of the liver and have found no unequivocal evidence in the literature that anaesthesia is necessary to obtain maximum *ATP* and *ADP* levels in this organ. Le Page (1946), however, has found some indication of a loss of rat-liver *ATP* when the whole animal was frozen without anaesthesia. To detect any changes in the absence of anaesthesia we have taken a group of six animals, three of which were treated with carbon tetrachloride. All were given an injection of nembutal (50 mg./kg. body wt.) and, when light surgical anaesthesia had been effected, the liver was rapidly removed and dropped into liquid air. In all cases the *TCA* extracts and the corresponding barium-insoluble fractions gave analytical figures which confirmed the previous findings; hence no decomposition of the labile phosphate detectable by our methods occurs in the liver of guinea pigs when the animals are killed without anaesthesia.

The estimation of the N : 7 min.-labile P : pentose ratios has been carried out by Le Page & Umbreit (1945) and Le Page (1946) to establish the identity of the adenosine polyphosphates in the barium-insoluble fraction. Our results, with similar methods, on guinea-pig liver, confirm the opinions of these workers and indicate that a considerable fraction of the 7 min.-labile P has its origin in *ADP*. This conclusion is not supported by results obtained with the *ATP*-ase method. These were only few in number since we were concerned initially with quantitative changes in those compounds possessing high-energy phosphate bonds (~ph; Lipmann, 1941). As far as the determination of the identity of the compounds is concerned, it seems more likely that dependable results will be achieved with the specific *ATP*-ase than with chemical methods, which will be subject to error from possible contamination with N or P compounds. It is worthy of note that Rapoport (1945), using the *ATP*-ase method, has found *ATP* in livers of rats in amounts much greater than those found by Le Page & Umbreit (1945) and Le Page (1946).

The changes in the distribution of the acid-soluble phosphates in the fatty liver of the guinea pig, in so far as we have investigated them, indicate a marked increase in *APP* and phosphocreatine. In the interpretation of these results the work of Kaplan & Greenberg (1944*b*) is of interest, as these workers, using fasted and fat-fed rats, claimed a decrease in ~ph levels, attributed to the appropriation of ~ph for the oxidation of fat without a replacement being effected through the utilization of acetoacetate in the liver. It is doubtful, however, if such a hypothesis is tenable, since it assumes the obligatory formation of acetoacetate as the end product of fatty acid oxidation. This is not necessarily so as is indicated by numerous experiments with anti-ketogenic substances. An explanation for the non-appearance of acetoacetate may be found in the association of the citric acid cycle with fatty acid oxidation (cf. Quastel & Wheatley, 1935). Positive evidence for this is found in the action of the enzyme citrogenase, present in the liver (Breusch, 1943), which catalyzes the reaction between acetoacetate and oxaloacetate to form citrate (Wieland & Rosenthal, 1943; Buchanan, Sakami, Gurin & Wilson, 1945 *a*, *b*).

It is therefore suggested that the increased amount of ~ph which has been noted in our experiments arises from energy derived from the citric cycle as a direct consequence of the increased amounts of fat which are being oxidized.

It has been suggested by Lehninger (1945*b*) that ~ph might be generated by the stepwise oxidation of fatty acids down to the stage of acetoacetate. Experiments with malonate suggest that this process is unlikely to result in increases in ~ph. Thus the well known *in vivo* and *in vitro* effect of malonate in increasing acetoacetate production is generally assumed to be due to the denial of the citric acid cycle as one avenue for the oxidation of fat. Moreover, malonate has been shown by Kaplan & Greenberg (1944*a*) to produce a decrease in ~ph in the rat liver. It would seem, therefore, that the increases in ~ph which we have observed are due not to the reactions involved in the degradation of the fat down to the acetoacetate level, but to the reactions which occur following the entry of the acetoacetate into the citric acid cycle.

The increased amount of *APP* which we have observed in the fatty livers of guinea pigs is of interest in view of the work of Lehninger (1945*b*) on the role of *ATP* and *ADP* in fatty acid oxidation. In considering the implication of these results it should be remembered that Lehninger showed that, in crude rat-liver homogenates, the activation produced by *ADP* was equal to that by *ATP* when both were present in equimolar concentrations. Under these conditions there is twice the amount of ~ph available in the *ATP*-catalyzed system as in that catalyzed by *ADP*. When the molar concentration of *ATP* was reduced to one half that of *ADP*, thus providing equal amounts of ~ph, *ATP* produced only 70% of the activation provided by *ADP*. It does not seem that this can be explained by the assumption that the diphosphate is raised to the triphosphate stage by phosphorylation, since the addition of adenylic acid, which presumably would be phosphorylated if this were so, was without effect. The only conclusion to be drawn from these results is that *ADP* is at least as important as *ATP* to the *in vivo* systems responsible for the oxidation of fatty acids.

In later work Lehninger (1945*a*) employed purified homogenates and showed that although *ATP* produced the customary activation, *ADP* was inactive. Lehninger interpreted this as indicating the specific nature of the activation produced by *ATP*. While this is undoubtedly so in the system employed, the earlier results (Lehninger, 1945*b*) cannot be discounted. They suggest that, whilst the study of purified liver homogenates will undoubtedly give valuable evidence as to the precise nature of the activators of a particular system, the findings are not necessarily applicable without modification to the oxidation of fatty acids in the intact liver occurring in association with the oxidation of other substrates.

On thermodynamic grounds, the phosphorylation of *ADP* to *ATP* requires no greater utilization of energy than that of creatine to phosphocreatine. But it is of interest that the fatty liver, whilst containing greater amounts of ~ph (*APP* and phosphocreatine), does not contain exclusively *ATP* if complete reliance be placed upon the determination of the N : 7 min.-labile P : pentose ratios. Some doubt as to the reliability of these analyses arises when one considers the increases in phosphocreatine which have been demonstrated, since it is difficult to conceive of ~ph being transferred to creatine without a prior transfer to saturation point of the adenylic acid system, and with the consequent appearance of *ATP*. Further experiments with the more specific *ATP*-ase methods should settle this point.

It is well known that phosphocreatine is capable of phosphorylating adenylic acid, and Lehninger (1945*b*) has shown that the activation of fatty acid oxidation is greater when both adenylic acid and phosphocreatine are present than with phosphocreatine alone. The phosphocreatine found in the normal liver may then be regarded as representing a readily available source of energy, which can be utilized through the adenylic acid system when the occasion arises, and its presence in increased amounts in the fatty liver is most probably the result of an overflow of ~ph which cannot be stored as *APP*.

The continued failure to detect the presence of acyl phosphates in the fatty liver is disappointing in view of the accumulated evidence which suggests the necessity of phosphorylation as an integral part of the fatty acid oxidation mechanism. That acyl phosphates may be oxidized in the absence of added *ADP* or *ATP* (Lehninger, 1944) is interesting but is not conclusive proof of the intermediate formation of these compounds, since they themselves are capable of phosphorylating adenylic acid with the formation of *ATP*.

SUMMARY

1. The change in the distribution of acid-soluble phosphates in the fatty livers of guinea pigs treated with carbon tetrachloride has been investigated.

2. In such livers there is an increase in the total acid-soluble organic phosphates.

3. This increase is considered as primarily due to increases in adenosine polyphosphates and phosphocreatine, and in general runs parallel to the fat content.

4. Acyl phosphates have not been detected in liver extracts.

5. The results are interpreted in the light of modern work on fatty acid oxidation.

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REFERENCES

Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
Breusch, F. L. (1943). *Science*, **97**, 490.
Buchanan, J. M., Sakami, W., Gurin, S. & Wilson, D. W. (1945*a*). *J. biol. Chem.* **157**, 747.
Buchanan, J. M., Sakami, W., Gurin, S. & Wilson, D. W. (1945*b*). *J. biol. Chem.* **159**, 695.
Campbell, W. R. & Hanna, M. I. (1937). *J. biol. Chem.* **119**, 1.
Conway, E. J. (1942). *Biochem. J.* **36**, 655.
Cori, C. F. & Goltz, H. L. (1925). *Amer. J. Physiol.* **72**, 256.
Eggleton, P., Elsden, S. R. & Gough, N. (1943). *Biochem. J.* **37**, 526.

Ennor, A. H. (1942). *Aust. J. exp. Biol. med. Sci.* **20**, 73.
Ennor, A. H. & Stocken, L. A. (1947). *Nature, Lond.*, **159**, 308.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Fiske, C. H. & Subbarow, Y. (1929). *J. biol. Chem.* **81**, 629.
Flock, E., Bollman, J. L. & Mann, F. C. (1936*a*). *J. biol. Chem.* **115**, 179.
Flock, E., Bollman, J. L. & Mann, F. C. (1936*b*). *J. biol. Chem.* **115**, 201.
Higgins, G. M., Berkson, J. & Flock, E. (1932). *Amer. J. Physiol.* **102**, 673.
Higgins, G. M., Berkson, J. & Flock, E. (1933). *Amer. J. Physiol.* **105**, 177.
Kalckar, H. M. (1945). *Ann. Rev. Biochem.* **14**, 283.
Kaplan, N. O. & Greenberg, D. M. (1943). *J. biol. Chem.* **150**, 479.
Kaplan, N. O. & Greenberg, D. M. (1944*a*). *J. biol. Chem.* **156**, 525.
Kaplan, N. O. & Greenberg, D. M. (1944*b*). *J. biol. Chem.* **156**, 543.
Kaplan, N. O. & Greenberg, D. M. (1944*c*). *J. biol. Chem.* **156**, 511.
Kosterlitz, H. W. & Ritchie, C. M. (1943). *Biochem. J.* **37**, 181.
Lehninger, A. L. (1944). *J. biol. Chem.* **154**, 309.
Lehninger, A. L. (1945*a*). *J. biol. Chem.* **161**, 437.
Lehninger, A. L. (1945*b*). *J. biol. Chem.* **157**, 363.
Le Page, G. A. (1946). *Amer. J. Physiol.* **146**, 267.
Le Page, G. A. & Umbreit, W. W. (1945). In *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*, by W. W. Umbreit, R. H. Burris & J. F. Stauffer. Minneapolis: Burgess Publishing Co.
Lipmann, F. (1941). In *Advances in Enzymology and Related Subjects*, **1**, 99, ed. by Nord, F. F. & Werkman, C. H. New York: Interscience Publishers Inc.
Lipmann, F. & Tuttle, L. C. (1944). *J. biol. Chem.* **153**, 571.
Lipmann, F. & Tuttle, L. C. (1945). *J. biol. Chem.* **159**, 21.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Meier, R. & Thoenes, E. (1933). *Arch. exp. Path. Pharmak.* **169**, 655.
Meijbaum, W. (1939). *Hoppe-Seyl. Z.* **258**, 117.
Munoz, J. M. & Leloir, L. F. (1943). *J. biol. Chem.* **147**, 355.
Nelson, N., Rapoport, S., Guest, G. M. & Mirsky, I. A. (1942). *J. biol. Chem.* **144**, 291.
Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
Quastel, J. H. & Wheatley, A. H. M. (1935). *Biochem. J.* **29**, 2773.
Rapoport, S. (1945). *J. biol. Chem.* **161**, 429.
Rapoport, S., Leva, E. & Guest, G. M. (1943*a*). *J. biol. Chem.* **149**, 57.
Rapoport, S., Leva, E. & Guest, G. M. (1943*b*). *J. biol. Chem.* **149**, 65.
Rapoport, S. & Nelson, N. (1945). *J. biol. Chem.* **161**, 421.
Stone, W. E. (1943). *J. biol. Chem.* **149**, 29.
Wieland, H. & Rosenthal, C. (1943). *Liebigs Ann.* **554**, 241.

The Estimation of Creatine

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During the course of an investigation into the metabolism of fatty livers (Ennor & Stocken, 1948) it became desirable to identify, and subsequently to estimate, amounts of a very labile phosphorus compound present in the livers of normal guinea pigs and in others treated with carbon tetrachloride. Although phosphate liberated from organic phosphate compounds in acid molybdate is often considered to arise from phosphocreatine, there are other possibilities, and we endeavoured therefore to correlate the labile phosphorus with the creatine liberated following mild hydrolysis. It was decided to estimate the creatine as such rather than after conversion to creatinine, and an attempt was made to employ the method of Eggleton, Elsden & Gough (1943). These authors have pointed out that trichloroacetic acid (*TCA*) extracts of liver contain inhibitory substances which prevent the estimation of creatine by the Barritt (1936) modification of the Vosges & Proskauer (1898) reaction. The suggestion was also made that these inhibitors were the ω-amino aliphatic acids.

In the fractionation of *TCA* extracts of guinea-pig liver by means of barium (Le Page & Umbreit, 1945) it was found that both the inhibitor and phosphocreatine were precipitated in the ethanol-insoluble fraction. An examination of this fraction indicated that the inhibitory substance or substances was more likely to be glutathione than an amino-acid as suggested by Eggleton *et al.* (1943). Such a supposition, because of the wide distribution of the tripeptide, necessarily casts some doubt upon the validity of the application of the α-naphthol—diacetyl reaction to the estimation of creatine in tissues.

This communication is concerned with some experiments on the inhibition of the Barritt reaction by sulphydryl compounds and suggests a modification of the procedure of Eggleton *et al.* by means of which a more accurate determination of the creatine content of tissues may be made.

EXPERIMENTAL

Reagents

Creatine hydrate. An A.R. specimen was recrystallized according to Hunter (1928) and its purity checked by N analysis.

Diacetyl. A stock solution of approx. 1·% was prepared from A.R. dimethylglyoxime as described by Walpole (1911). This was diluted and used as described by Eggleton *et al.* (1943). No apparent loss in activity has been observed following 3 months' storage at −1°.

α-Naphthol. A faintly coloured A.R. sample has been used throughout. It was considered unnecessary to effect further purification by steam distillation. The solution of this compound and the volume used was, in all cases, as described by Eggleton *et al.* (1943).

Glutathione (GSH). This was a commercial specimen.

Oxidized glutathione (GSSG). This was prepared by bubbling O_2 through a slightly alkaline solution of GSH of appropriate concentration until a negative nitroprusside reaction was obtained.

Benzyl mercaptan and methionine. These were gifts from the Ministry of Supply.

Cysteine ester hydrochloride. This compound was mentioned casually by Friedmann (1903) who gave no details of the synthesis. A mixture of 20 g. cystine, 60 ml. conc. HCl (36·5 % w/v), 20 ml. water, 200 ml. ethanol and 20 g. tin foil were gently refluxed until all the tin had dissolved. After removal of the tin with H_2S, the solution was evaporated to dryness *in vacuo* and the residue dissolved in 150 ml. ethanol and saturated with dry HCl at 0°. Crystals of cysteine ester hydrochloride separated on standing overnight. Yield 24 g. (79 %). M.p. 128°. SH by I_2 titration, 17·7 %; calc. for $C_5H_{12}O_2SNCl$, 17·8 %.

Cystine ester hydrochloride. This was prepared according to the method of Friedmann (1903).

p-Chloromercuribenzoic acid was synthesized according to Whitmore & Woodward (1932).

2:3-*Dimercaptopropanol (BAL)* was obtained as described by Stocken (1947).

Procedure

This, apart from one minor modification, has followed the method described by Eggleton *et al.* (1943). Greater reproducibility is obtained if, after adjusting the volume of the creatine-α-naphthol-diacetyl solution to 10 ml. in a narrow tube, the contents are transferred to a 6 × 1 in. tube for mixing and development of the colour. After 30 min. development at room temperature (18°) the intensity of the colour was measured in 1 cm. cells in the Hilger Spekker absorptiometer with Ilford filter no. 604 (spectrum green).

Influence of monothiols on the Barritt reaction

Cysteine ester hydrochloride. For preliminary tests and because of the small amount of GSH available, the initial experiments were carried out with cysteine ester hydrochloride. Varying amounts of this compound were added to tubes each containing 1·0 ml. of 5×10^{-4}M-creatine (65·5 μg.). The percentage inhibition of colour development was then calculated and plotted against the molar ratio, cysteine ester : creatine.

The influence of cysteine ester on the colour produced by creatine, diacetyl and α-naphthol is very marked, 60 % inhibition being observed with a molar ratio of cysteine ester : creatine of 0·8 : 1.

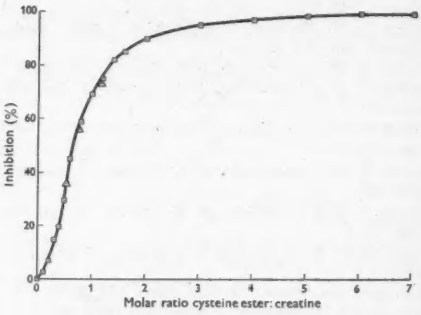

Fig. 1. Inhibition of the Barritt reaction by varying amounts of cysteine ester hydrochloride and glutathione. 65·5 μg. of creatine throughout. To all tubes were added 2·0 ml. α-naphthol solution (1·0 g. α-naphthol in 100 ml. of a solution containing 6·0 g. NaOH + 16 g. Na_2CO_3) + 1·0 ml. of a 1 in 20 dilution of stock diacetyl + varying amounts of thiol. Adjusted to 10 ml. with water and colour measured after 30 min. at room temperature. ⊡ denotes cysteine ester hydrochloride; △ denotes glutathione.

Glutathione (GSH). To test the inhibitory effect of GSH three points on the cysteine ester curve were chosen and the colour produced by creatine in the presence of a GSH : creatine ratio of 0·5, 0·8 and 1·2 mol. to 1 was determined. The points marked △ in Fig. 1 were obtained in this manner and are sufficiently close to the cysteine-ester inhibition curve to suggest that it is primarily the thiol group which is responsible for the inhibition of the Barritt reaction.

Benzyl mercaptan and 2:3-*dimercaptopropanol.* Two simple thiols were used in an attempt to show that the thiol group is the determining factor in the inhibition of the colour.

Table 1. *Inhibition of Barritt reaction by simple thiols*

Molar ratio thiol : creatine	Inhibition (%) Benzyl mercaptan	BAL
0·5	47	11·5
0·8	66	17·5
1·2	84·5	24·0
2·0	—	46·5

The results (Table 1) obtained with benzyl mercaptan are similar to those found with cysteine ester and GSH and support the idea that the inhibition seen with the latter compounds is due primarily to the reactivity of the thiol group and not to the amino group. Further confirmation of this is given in the section dealing with the neutralization of the inhibitor. It will be noticed that the degree of inhibition given by BAL is very much less than that produced by the other compounds. It is likely that the inhibition is due to addition of the thiol group across some reactive double bond formed in the sequence of reactions leading to the coloured complex. If this is the case, it is not to be expected that BAL would be as efficient an inhibitor as benzyl mercaptan even if it be supposed that only one of the thiol groups in BAL is capable of such an addition.

It is clear from the foregoing experiments that thiol-containing compounds present in tissues will produce considerable interference with the estimation of creatine, and that the magnitude of the error introduced will depend on the ratio of thiol to creatine. Thus, in those tissues where the creatine content is low and the thiol content high, it would be anticipated that the method discussed would not even detect creatine.

Effect of other sulphur-containing compounds

It was considered unlikely that the oxidation of the SH group would eliminate the inhibition because of the well known tendency towards cleavage of the disulphide linkage in alkaline solution. Experiments with GSSG and cystine ester (Table 2) confirm this, although it will be noticed that GSSG produces a much greater degree of inhibition, and is apparently, therefore, much more susceptible to hydrolysis than is cystine ester.

Table 2. *Disulphide inhibition of the Barritt reaction*

Molar ratio disulphide : creatine	Inhibition (%) Cystine ester	GSSG
0·25	—	22·0
0·4	—	34·0
0·6	—	50·0
1·0	3·4	—
2·0	5·0	81·0
4·0	9·1	—
8·0	18·1	—
16·0	31·9	—

It is clear from these experiments that not only thiols, but any compound which generates a thiol on treatment with alkali, will seriously interfere with the estimation of creatine in tissue extracts. It is to be regretted that the only other sulphur compound of biological importance which we have had the opportunity of testing in this reaction was methionine. This produced an inhibition of about 5 % when present in a molar ratio of 4 methionine : 1 creatine.

Protection of the Barritt reaction from thiol inhibition

It is apparent that when the molar ratio of SH to creatine exceeds 0·2–0·4 (7–15 % inhibition), the successful application of the Barritt reaction will depend on the removal of SH groups. Compounds known to combine with thiol groups include iodoacetic acid, maleate, trivalent arsenicals and organic mercurials. Of these we preferred the mercurials because of the high rate of formation and stability of the resultant *R*-Hg-S*R'* compound in alkaline solution. *p*-Chloromercuribenzoic acid was chosen as a possible protective agent in order to ensure non-precipitation of the Hg derivative.

Procedure. 1·0 ml. of a 5×10^{-4} M-solution of creatine (65·5 μg.) was added to a series of tubes, followed by 1·0 ml. of 4×10^{-3} M-cysteine ester. This gave an initial ratio of cysteine ester : creatine of 8 : 1 (a ratio of 7 : 1 will produce 100 % inhibition under these conditions, see Fig. 1). Varying volumes of an appropriate dilution of a 10^{-2} M-solution of *p*-chloromercuribenzoic acid (*CMBA*) were then added to produce *CMBA* : cysteine ester ratios from 0 to 2·0. α-Naphthol and diacetyl were then added as before and the resultant colours estimated after 30 min. All determinations were carried out in duplicate and the figures in Table 3 are the means of the values obtained.

Table 3. *Protection afforded by p-chloromercuribenzoic acid (CMBA) on the thiol inhibition of the Barritt reaction*

(Degree of protection = μg. creatine found/μg. creatine present × 100.)

Molar ratio *CMBA* : cysteine ester	Degree of protection
0·1	0
0·2	1·5
0·3	2·0
0·4	2·8
0·5	2·9
0·6	5·4
0·7	11·8
0·8	31·5
0·9	73·7
1·0	89·6
2·0	92·2

Increasing amounts of *CMBA*, as would be expected, lead to an increased removal of the inhibition produced by cysteine ester (Table 3). Complete removal of the inhibition, however, was not obtained even when the molar ratio of *CMBA* : cysteine ester was 2 : 1. Any of the three following factors could be responsible for this effect: (*a*) dissociation of the resultant *R*-Hg-S*R'*, (*b*) the *R*-Hg-S*R'* itself, (*c*) the excess of the *CMBA*.

The degree of protection afforded by *CMBA* against the effects of GSH, GSSG, benzyl mercaptan and BAL was similar to that obtained in the case of cysteine ester.

Effect of ethyl α-amino-β-(p-carboxyphenylmercurithio)-propionate and p-chloromercuribenzoic acid on the Barritt reaction. The ethyl α-amino-β-(*p*-carboxyphenylmercurithio)-propionate was not isolated, but prepared in solution by mixing equivalent amounts of *p*-chloromercuribenzoic acid and cysteine ester. Convenient amounts of suitable dilutions were then added to 1·0 ml. 5×10^{-4}M-creatine (65·5 μg.). The subsequent procedure was as described earlier.

Table 4. *Inhibition of the Barritt reaction by p-chloromercuribenzoic acid (CMBA) and ethyl α-amino-β-(p-carboxyphenylmercurithio)-propionate (R-Hg-SR′)*

Molar ratio compound : creatine	Inhibition (%)	
	R-Hg-S*R′*	*CMBA*
0·5	0	0
1·0	0	0
2·0	0	1·7
4·0	4·9	4·5
8·0	5·3	6·1
10·0	—	7·4
16·0	8·2	—
32·0	11·1	—

The results of this experiment (Table 4) indicate that some inhibition is to be expected when the ratio of either the complex, or of the *CMBA*, to creatine exceeds 2 : 1. Since, however, a ratio of 4 : 1 only produces an inhibition of 5% and a ratio of 2 mol. *CMBA* to 1 mol. cysteine affords 92% protection, it is clear that the error introduced is small compared with that obtained when free thiol compounds are present.

Stoicheiometric relationship of the cysteine ester inhibition. It was considered worth while to determine the colour developed by varying amounts of creatine in the presence of varying amounts of cysteine ester when the relative amounts of each were maintained at a constant ratio. A molar ratio of cysteine ester to creatine of 0·6 : 1 was chosen since at this value the inhibition expected is 44·8% (Fig. 1).

The strictly linear relationship between the amount of creatine and the optical density is shown in Fig. 2. In the presence of cysteine it will be noticed that there is a distinct diminution in the inhibition at low concentrations of the thiol (Fig. 3).

If it is true that interference with the production of the coloured compound is due to a preferential reaction of some intermediate compound with the thiol then it would be expected, under the conditions of this experiment, that the percentage inhibition would remain constant. This, however, is not the case, and the explanation is probably to be found in the small absolute amount of cysteine ester which is present at the lower creatine levels. Thus at 8·18 μg. of creatine there are only 5·6 μg. of cysteine ester in a volume of 10·0 ml., and it must be considered unlikely that such a small amount would remain in the reduced state. The actual ratio of cysteine ester to creatine would thus be less than 0·6 : 1. Reference to Fig. 1 shows that a reduction of this ratio to 0·4 : 1 would account for the low inhibition seen at the 8·18 μg. level of creatine.

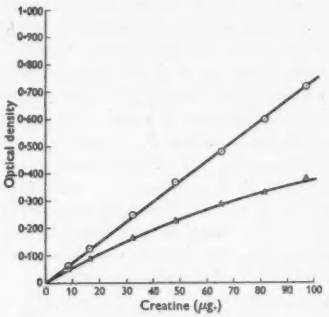

Fig. 2. Comparison of the colour produced by varying amounts of creatine in the absence ⊙ and the presence △ of a molar ratio of cysteine ester : creatine of 0·6 : 1.

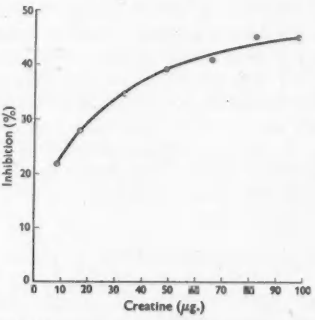

Fig. 3. Variation in the inhibition of the Barritt reaction in the presence of a constant ratio of cysteine ester : creatine of 0·6 : 1.

Application of the method to the estimation of creatine in tissue extracts

Well fed, adult, male rats of about 300 g. body weight were stunned by a blow on the head and the neck vessels severed. Samples of the various tissues were weighed in a mortar of the Potter & Elvehjem (1936) type, and homogenized with 5 ml. of 10% (w/v) trichloroacetic acid (*TCA*). After centrifugation, the residue was re-extracted with 10 ml. of 5% *TCA*. The combined supernatant fluids were made up to 25·0 ml. with water and filtered prior to the removal of samples for estimation.

Procedure. The creatine was determined in suitable samples of the *TCA* extracts both in the presence and absence of *CMBA*. To obtain some idea of the absolute amounts of reducing substances present, and hence the amount of *CMBA* necessary to combine with the SH present, samples of the *TCA* extracts were titrated with 0·01 N-iodine after the addition of 0·5 vol. N-HCl (to facilitate comparison with the previous experiments, the iodine titres are expressed as equivalents of cysteine ester). If it be assumed that the reducing substances are entirely thiol in character the addition of an equivalent amount of *CMBA* will ensure an excess of the reagent. The figures quoted in Table 5 are the means of at least two determinations on each sample.

Table 5. *Creatine content of rat tissues found in the presence and absence of CMBA*

Tissue	Cysteine ester equivalent (mg./100 g.)	Creatine (mg./100 g.) *CMBA* absent	Creatine (mg./100 g.) *CMBA* present	Molar ratio cysteine ester equivalent : creatine	Increase with *CMBA* (%)
Liver (1)	185	0	4·6	28·8	∞
(2)	244	0	3·8	45·7	∞
Spleen (1)	161	5·9	15·8	7·2	197
(2)	181	3·9	15·3	8·4	292
Kidney (1)	139	7·7	20·5	4·8	166
(2)	159	9·2	22·9	4·9	150
Lung (1)	124	6·0	13·0	6·8	117
(2)	149	5·8	11·8	9·0	104
Smooth muscle (small intestine) (1)	124	24·2	40·4	2·2	67
(2)	147	20·7	34·8	3·0	68
Testis (1)	172	248·0	295·0	0·4	16·5
(2)	178	260·0	299·0	0·4	15
Brain (1)	84	114·5	130·0	0·5	14
(2)	136	115·0	133·0	0·7	15
Cardiac muscle (ventricle) (1)	110	170·0	192·0	0·4	13
(2)	147	197·0	212·0	0·5	7
Skeletal muscle (1)	46	516·0	520·0	0·06	2
(2)	39	500·0	511·0	0·05	2

It will be noticed that in the case of kidney, spleen, lung and small intestine, the addition of *CMBA* produced a considerable increase in the amount of creatine estimated (Table 5). In the case of liver, the addition of *CMBA* now renders possible the determination of the creatine present, zero values being recorded in its absence. With the remaining tissues investigated, voluntary muscle, testis, brain and cardiac muscle, the increases are not as great, a maximum mean increase of only 15·7 % being present in the case of testis.

The magnitude of the increase in the amount of creatine measurable in the presence of *CMBA* will depend on the relative amounts of thiol and creatine. The method employed to obtain the cysteine ester equivalent, and thus the ratio of this to creatine, will lead to a high result. Nevertheless it is of some interest to note that there is a fair correlation between the ratios so obtained and the percentage increase in the creatine measured in the presence of *CMBA*. Thus small intestine, kidney, lung and spleen with ratios (obviously too high, cf. Fig. 1), of 2·6–7·5 : 1 show increases from 70 to 250 %, and the other tissues with ratios of 0·05–0·5 : 1 have increases of 2–16 %. In the case of liver tissue, a mean ratio of 37·2 : 1 will prevent the estimation of creatine in the absence of *CMBA* and thus the addition of this compound must lead to an infinite increase.

Although the figures for the creatine content of the various tissues examined are the maximum obtainable in the presence of *CMBA*, they do not necessarily represent the true creatine values because of the possible inhibition by substances other than the *R*-Hg-S*R′* compound formed and the excess *CMBA* present. This possibility was examined by attempting to recover 20 μg. of creatine which were added to samples before analysis.

Appropriate samples of the *TCA* extracts were taken in quadruplicate, and to each set of tubes 2·0 ml. of 5×10^{-2} M-*CMBA* (1426 μg.) were added. To two of the tubes in each series 20 μg. of creatine were added. α-Naphthol and diacetyl were added as before and the colour measured after 30 min. In view of the inhibition produced by the excess of *CMBA*, the amount of creatine was obtained by reference to a standard curve prepared by measuring the colour produced by known amounts of creatine in the presence of 1426 μg. of *CMBA*.

The recoveries of 20 μg. of added creatine (Table 6) indicate that this procedure will give values for the creatine contents of tissues which are reasonably close to those actually present.

Table 6. *Recovery of 20 μg. creatine added to trichloroacetic acid extracts of various rat tissues in the presence of CMBA*

Tissue	Creatine found (μg.) No addition	Creatine found (μg.) +20 μg. creatine	Recovery of added creatine (%)
Muscle	28·4	48·4	100
Testis	18·0	37·4	97
Lung	13·6	31·6	90
Brain	22·2	41·0	94
Cardiac muscle (ventricle)	20·2	41·0	104
Liver	18·2	36·2	90
Spleen	13·2	30·8	88
Kidney	43·4	60·8	87
Smooth muscle (small intestine)	41·3	59·9	93

As a matter of interest the behaviour of the α-naphthol-diacetyl method for the estimation of creatine in whole blood has also been studied (Table 7).

liver extracts and in urine. They further state that extracts of most animal tissues, with the exception of liver, 'appear to contain insignificant amounts of interfering substances'. An examination of the ethanol-insoluble barium salts obtained from *TCA* extracts of liver led us to the conclusion that GSH was more likely to be the agent responsible for the inhibitory effect, and further that it is the SH group which is the determining factor.

The inhibitory influence of SH-containing compounds on the α-naphthol—diacetyl reaction for the estimation of creatine is very striking and depends on the molar ratio of SH : creatine. In the estimation of creatine in spleen, kidney, lung and intestine, the SH : creatine ratio is sufficient to produce errors ranging from 60 to 250 %.

The inhibitory action of SH can be removed almost completely by the addition of *p*-chloromercuribenzoic acid, and the figures obtained in the presence of this compound suggest that the method, so modified, is now capable of giving creatine values

Table 7. *Creatine content of whole blood found in the presence and absence of CMBA*

(10·91 g. of rabbit blood + 10 ml. of 10 % (w/v) trichloroacetic acid, proteins separated by centrifugation. The insoluble protein residue re-extracted with a further 10 ml. of 5 % trichloroacetic acid, centrifuged and the supernatant fluids combined and made up to 25·0 ml. with water. Filtered. 2·0 ml. samples taken in all cases.)

Creatine in sample (μg.) *CMBA* absent	*CMBA* present	+20 μg. creatine, *CMBA* present	Increase in presence of *CMBA* (%)	Recovery (%)
14·6	18·6	36·2	27·4	88

Creatine, mg./100 g. blood = 2·4 (calculated from creatine measured in sample in the presence of *CMBA*).

Here again the recovery of added creatine may be carried out satisfactorily and is comparable in magnitude with that observed in tissue extracts. The influence of SH is again well illustrated by the 27 % increase in the amount of creatine measured in the presence of added *CMBA*.

Some experiments have been carried out with urine from normal males, young children and pregnant females. These have been neither sufficiently numerous nor comprehensive to quote in detail, but some of the preliminary results are of interest. It has been found that some degree of inhibition is present in 1·0 ml. of urine as judged by the increased values in the presence of *CMBA*. These increases were variable and have not been correlated with other factors, but were of the order of 10 %. 100 % recoveries of added creatine were obtained by the addition of *CMBA*, and it therefore appears possible that this modification may prove useful for the estimation of creatine in urine.

DISCUSSION

It has been suggested by Eggleton *et al.* (1943) that ω-amino-acids may be responsible for the failure of the Barritt reaction to estimate creatine in *TCA*-

which are close to the true values. An effect, similar to that obtained with this substance, could probably be gained by the addition of other organic mercurials and, in fact, by any substance reacting with SH groups to give a complex stable to alkali; but since *p*-chloromercuribenzoic acid behaved satisfactorily in our hands this has been used exclusively.

The results of experiments with pure SH compounds and SH-containing tissue extracts in the presence of *p*-chloromercuribenzoic acid strongly suggest that SH compounds are the only inhibitors of importance as far as the application of the Barritt reaction to tissue extracts is concerned. This conclusion is further strengthened by the fact that, in most instances, recoveries of known amounts of added creatine were 90 % or greater. Although Eggleton *et al.* (1943) have observed a 50 % reduction of the colour produced by 30 μg. of creatine in the presence of 4 mg. of glycine it seems improbable that such amounts of any amino-acid could be present in the usual samples taken for estimation of creatine in *TCA*-tissue extracts.

The only body fluids examined have been whole blood and urine, and in both cases the addition of *CMBA* appears to overcome the inhibitors which

are present. The suggestion of Walpole (1911) that it is the NH_3 which interferes with the reaction in normal urine has not been confirmed.

It follows from the high degree of sensitivity of this reaction to the presence of sulphydryl compounds that use might be made of this fact for the detection, and possibly the estimation, of small amounts of SH and alkali-labile SS substances. Experiments are in progress to test this possibility.

As Barritt (1936) has pointed out, the reaction between diacetyl, α-naphthol and creatine to form a coloured product is also given by other guanido compounds. Eggleton *et al.* (1943) have stated that arginine, guanidine and glycocyamine produce about one ninth of the colour produced by equivalent amounts of creatine. All results quoted in this communication have been calculated on the assumption that creatine alone produces the colour measured. It is believed that the error introduced by such an assumption is small. However, in the application of the revised method, the possibility of increased amounts of other guanido compounds in pathological states must be borne in mind.

SUMMARY

1. The α-naphthol—diacetyl reaction for the estimation of creatine has been investigated.

2. The inhibition, which has been noted by other workers to occur when the estimation of the creatine content of trichloroacetic acid extracts of liver is attempted, has been shown to be due to the presence of sulphydryl compounds and, by inference, to the presence of glutathione.

3. The inhibition by sulphydryl compounds depends on the molar ratio of the sulphydryl compound to creatine, and is therefore most marked in the liver where the creatine content is low and the glutathione content high.

4. The ratio of sulphydryl compounds to creatine, in several tissues investigated, is sufficiently high to produce very gross errors in the estimation of creatine by this method. Exceptions are found in voluntary and cardiac muscle, brain and testis, but even here the method is subject to error.

5. The presence of disulphide compounds such as GSSG also produces an inhibition due, presumably, to the tendency of the disulphide linkage to split in alkaline solution.

6. The inhibition produced by sulphydryl compounds can be almost completely removed by the addition of *p*-chloromercuribenzoic acid; both this compound and that formed by its reaction with the sulphydryl compounds possess weak inhibitory powers.

7. The creatine content of various tissues, blood and urine has been determined both in the presence and in the absence of *p*-chloromercuribenzoic acid and an assessment made of the errors introduced by the omission of this compound.

8. The recoveries of creatine added to extracts of various tissues, blood and urine are mostly in excess of 90%, thus indicating the general applicability of the modified method.

We wish to express our thanks to the Chief Scientist of the Ministry of Supply for gifts of methionine and benzyl mercaptan and to Messrs E. Facer and S. Moorbath for skilled technical assistance.

REFERENCES

Barritt, M. M. (1936). *J. Path. Bact.* **42**, 441.
Eggleton, P., Elsden, S. R. & Gough, N. (1943). *Biochem. J.* **37**, 526.
Ennor, A. H. & Stocken, L. A. (1948). *Biochem. J.* **42**, 549.
Friedmann, E. (1903). *Beitr. chem. Physiol. Path.* **3**, 1.
Hunter, A. (1928). *Creatine and Creatinine.* London: Longmans Green.
Le Page, G. A. & Umbreit, W. W. (1945). *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*, by W. W. Umbreit, R. H. Burnis & J. F. Stauffen. Minneapolis: Burgess Publishing Co.
Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
Stocken, L. A. (1947). *J. chem. Soc.* p. 592.
Vosges, O. & Proskauer, B. (1898). *Z. Hyg. InfektKr.* **28**, 20.
Walpole, G. S. (1911). *J. Physiol.* **42**, 301.
Whitmore, F. C. & Woodward, G. E. (1932). *Organic Synth.* Collective **1**, 153.

The Fermentation of Cellulose *in vitro* by Organisms from the Rumen of Sheep

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The suggestion that micro-organisms are responsible for the decomposition of cellulose in the alimentary canal (Wildt, 1874; Zuntz, 1879; Duclaux, 1882) arose from the proof (Henneberg & Stohmann, 1860–4) that it is, in fact, cellulose which disappears during the passage of crude fibre through the animal (Haubner, 1855). Tappeiner (1882, 1883, 1884, 1888), who was the first to investigate the fate of cellulose, found that paper pulp and cotton wool, when incubated under anaerobic conditions with the fluid contents of the rumen, dissolved slowly with the evolution of methane and carbon dioxide and the formation of fatty acids. He ascribed little nutritional value to the fatty acids, however, and suggested that the fermentation of cellulose was important only because it allowed the digestive enzymes access to the contents of the plant cells (Tappeiner, 1884). This view was not accepted (Henneberg & Stohmann, 1885); the fact that only traces of fatty acids appear in the excreta (Wilsing, 1885; von Knieriem, 1885) implies at least that they are absorbed and oxidized. There was already positive evidence of the nutritional value of cellulose (von Knieriem, 1885; Lehmann, 1889). Apart from the investigations of Markoff (1911, 1913) which added little to the information provided by Tappeiner (1884) this aspect of ruminant digestion was abandoned in a state of considerable confusion.

Tappeiner (1888) distilled the medium from somewhat prolonged *in vitro* fermentation of cellulose by rumen contents, and from the combining capacity and composition of the silver salts of the mixed volatile fatty acids in the distillate, claimed that acetic and butyric acids were the main products of fermentation. Quite apart from the possibility that his cultures had been qualitatively affected by the environment in which the fermentations were carried out, the chemical methods upon which the claim was based were not capable of distinguishing between mixtures of these two acids and any combination of the lower homologues. Examination of his data reveals no sound reason for the conclusion that only the 2-C and 4-C acids were among the products. On the occasion when he examined a series of fractions collected during progressive distillation Tappeiner (1884, p. 91) concluded, 'Zweifelhaft bleibt das Vorkommen von Propionsäure'. His data clearly indicated the presence of considerable quantities of propionic acid.

Markoff's cultures also were probably subject to environmental alteration, and apparently he was unaware that the higher homologues distil first from a mixture of fatty acids in aqueous solution, and that an analytical technique based on distillation constants had been published by Duclaux (1874, 1895, 1900). He concluded without sound reason that the average molecular weight of the fatty-acid products of his fermentations of cellulose was the same as that of butyric acid (Markoff, 1913, p. 36). There was thus no real justification for the assumptions which led Honcamp (1909, 1927) to derive empirical equations for the degradation of cellulose in the alimentary canal.

Before the experiments described here were started* we were aware that considerable quantities of propionic acid appeared in the rumen during fermentation, but acetic acid usually predominated and sometimes accumulated in greater excess over the propionic acid than would be expected even if the pentosans of the fodder were fermented preferentially (Seillière, 1910) to acetic acid. This fact was difficult to reconcile with our observations on the apparent overall usefulness to the animal of the products of rumen fermentation. It appeared from the work of Lusk and his colleagues (Ringer & Lusk, 1910; Lusk, 1921; Milhorat & Deuel, 1926–7) that, in the dog at least, acetic acid was rapidly and wastefully oxidized without entering the carbohydrate cycle; and it was obvious from energy transactions that the ruminant derives more useful energy from its fodder than would be implied if the greater proportion of the carbohydrates were converted to acetic acid and subsequently disposed of without entering into the productive channels of metabolism.

Although these *in vitro* fermentations were conducted under conditions as similar to those obtaining in the rumen as were technically feasible, a considerable preponderance of propionic acid accumulated. This finding was in more reasonable agreement with the results of some calorimeter experiments on sheep carried out in this laboratory, which we hope to publish shortly, but the (acetic)/(propionic) ratio was so different from that found in the rumen contents as to lead to a suspicion that the community of micro-organisms responsible for the fermentations *in vitro* had been so altered that the course of degradation had been changed materially from that which normally prevails in the paunch.

The confirmation by Barcroft and his colleagues of Trautmann's (1933) observation that absorption may take place directly through the rumen wall (Barcroft, McAnally & Phillipson, 1944*a*), and their convincing demonstration

* The experiments were carried out in part at the Biochemical Laboratory, Cambridge, during 1937 when the author was the guest of the late Sir Frederick Gowland Hopkins, and in part at the author's laboratory, Adelaide, in 1938.

that considerable quantities of fatty acids find their way into the blood stream draining the rumen (Barcroft *et al.* 1944 *b*, *c*), provided the clue which led to a more complete understanding of ruminant digestion and to a better appreciation of the energy transactions involved in the utilization of the fermentation products. Further experiments have shown that when the contents of the rumen are acid, as they normally are during active digestion, propionic acid is absorbed preferentially to acetic acid (Danielli, Hitchcock, Marshall & Phillipson, 1945; Gray, 1947). The relative rates of diffusion through the rumen wall are such as may normally reverse the ratio of acetic/propionic acids in the contents of the rumen. Conclusions based only on analyses of rumen contents may thus be misleading. The necessity for further knowledge of the products of *in vitro* fermentations conducted in an environment similar to that of the rumen is obvious.

The environment in the rumen

The environment in the rumen is the main factor which regulates the community of micro-organisms responsible for carbohydrate degradation. Outstanding features of this environment are:

(*a*) *Salivary secretion.* A relatively large quantity of dilute alkaline saliva is secreted continuously by the parotid glands.* Besides its buffering capacity and its ability to maintain a high CO_2 tension in the medium where the fermentation proceeds, the parotid saliva carries to the micro-organisms a supply of phosphate and simple nitrogenous compounds, which is in very considerable excess of the quantity usually available from the fodder.

(*b*) *The reaction of the rumen contents.* When in a quiescent state, 24 hr. after ingestion of food, the rumen contents are about neutral. In samples from twelve animals taken at slaughter and collected without loss of CO_2, the reaction at 40° ranged between pH 6·40 and 7·14, with a mean of pH 6·8. The fatty acids produced during fermentation are in part neutralized by the saliva, and in part removed either by passage to lower levels of the alimentary canal or by direct diffusion through the rumen wall. When fermentation is most vigorous, during the period between 4 and 8 hr. after feeding, acid production exceeds these modifying influences; the mass then becomes distinctly acid, sometimes less than pH 5.

(*c*) *The oxidation-reduction condition of the liquid contents and the gas tension of the atmosphere above.* The partial pressure of oxygen is usually less than 8% in the atmosphere above the liquid contents of the quiescent rumen 24 hr. after feeding. This is augmented temporarily by the oxygen in the air swallowed with the food. It is diminished abruptly to very low levels (cf. Washburn & Brody, 1937), mainly through displacement by the CO_2 and the methane evolved when active fermentation begins soon after feeding. The liquid contents of the rumen, however, maintain a low oxidation-reduction potential and, even after the animal has fasted 3 days, the conditions are such as to allow some methane production to proceed. Normally there is a high partial pressure of CO_2 in the rumen gas at all times.

Experimental imitation of the milieu

The low partial pressure of oxygen and the high CO_2 tension, the supply of phosphate and inorganic nitrogen from the saliva, the buffering capacity of the saliva, the temperature of approximately 40°, and the residual contents of the rumen, which retain a massive inoculum of a community of micro-organisms already selected by the *milieu*, are apparently the main factors which determine the course of fermentation in the normal rumen. When subjected to these conditions, the complex carbohydrates and simple sugars are fermented very rapidly. Some observations (unpublished) on the rate of disappearance of cellulose from different levels of the intestinal tract of sheep indicated that a digestion mass of approximately 5 l. in the rumen of a sheep fermented in 8 hr. from 400 to 600 g. of mixed carbohydrates, at least half of which was cellulose, and that the rate of dissimilation at the peak of activity exceeded considerably the average over this period. The rapid rate of fermentation and of absorption of the products was reflected in the energy metabolism of the sheep: after feeding with 1 kg. of hay the metabolic rate increased within an hour to a level approximately 25% above that previous to feeding, remained there for 6–7 hr., and then receded during the ensuing 2 hr. to the lower level which was maintained until the animal was fed again 24 hr. later.

* Colin (1886) estimated that approximately 60 l. of parotid saliva are secreted each day by the ox. Scheunert & Trautmann (1921) concluded that the sheep secretes approximately 4 l. in 24 hr., which is probably a low estimate. In an investigation preliminary to these *in vitro* fermentation experiments, the author established fistulae in the left parotid ducts of three mature ewes and, when the secretion was passed back into the mouth at frequent intervals, collected up to 3·5 l. of saliva in 24 hr., which would imply the secretion of approximately 7 l. each day under the particular feeding conditions of the experimental animals. They were fed at noon each day with 1·2 kg. of a mixture of chaffed cereal and lucerne hays, and consumed the whole of it within 2 hr. (cf. Marston, 1939). In these studies there was evidence of reflex stimulation by the acidity of the rumen contents, and so a reasonable estimate was possible only if the secretion was collected during the whole cycle of 24 hr. Chemical analyses of the saliva confirmed Scheunert & Trautmann's (1921) estimate of the bicarbonate concentration (equivalent to 0·1–0·2 N-$NaHCO_3$) and indicated that both the phosphate and the urea, which became concentrated in the secretion, reflected changes in the inorganic phosphate and urea concentrations of the blood. Watson (1933), in this laboratory, had established previously that the concentration of phosphate in the blood was related to that of the mixed saliva collected on sponges inserted into the mouth.

EXPERIMENTAL

Apparatus. The vessel in which the fermentations were carried out was a 3·5 l. glass pot, hermetically sealed with a metal lid clamped down on a rubber gasket (Fig. 1). A stirrer attached to a geared variable-speed motor which could at will be run very slowly, passed through a mercury-sealed bearing in the lid. Six ports, tapered to fit standard

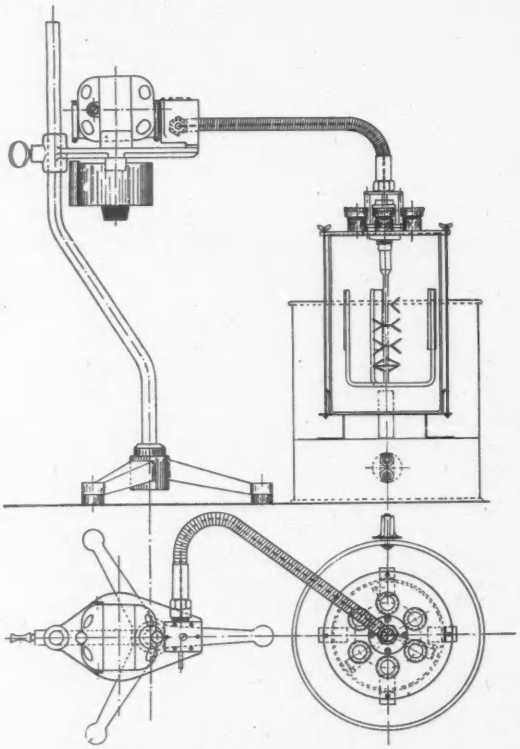

Fig. 1. Fermentation apparatus consisting of a 3·5 l. closed jar with mercury-sealed stirrer and six ports through which subsidiary apparatus was introduced.

rubber stoppers, through which other pieces of apparatus could be introduced, were attached to the lid. All metal parts within the pot were coated with paraffin wax. The subsidiary apparatus (Fig. 2) consisted of: an inlet and an outlet tube for gas, *A*, one of which was constructed to take the delivery point of a burette through which the standard alkali could be introduced; a standardized glass electrode for pH measurements, *B*; a bright platinum electrode, *C*, for oxidation-reduction potential measurement; a saturated calomel half cell, *D*, to provide a reference potential; a closed pipette, *E*, into which a sample of the liquid contents of the pot could be drawn up around a second glass electrode which was used on occasions to check the accuracy of the glass electrode, *B*; and a toluene-mercury thermoregulator which, through a relay, operated the heating elements of the water bath in which the pot was immersed. The transfer of heat through the pot was not sufficiently rapid to ensure very precise temperature regulation: the contents varied $\pm 1{\cdot}5°$ about the 40° setting.

The ingoing stream of N_2 was purified by passing over soda-asbestos and then over freshly reduced Cu maintained at approximately 600° in a silica-tube furnace. The outgoing stream, after dilution with approximately twice its volume of CO_2-free O_2, was dried by passing through H_2SO_4 and then over anhydrous $Mg(ClO_4)_2$ (Dehydrite) and the CO_2 absorbed with soda-asbestos (Ascarite) in two vessels placed in sequence. These were large enough to deal efficiently with the considerable volume of CO_2 evolved as a fermentation product, and from the acid decomposition of the Na_2CO_3 which was introduced during the digestion. Guard tubes containing anhydrous $Mg(ClO_4)_2$ were introduced into the chain at each point immediately after the CO_2 absorbers. The dry, CO_2-free gas was then led through the combustion chain which is described in a later section.

Substrate, culture medium and inoculum. The cellulose used in Exps. 1 and 2 was a specimen provided by Dr David Bell and described by him as 'practically pure α-cellulose from birch wood'; in Exps. 3 and 4 it was prepared from filter paper (Munktell 1 F) which had been suspended in 17·5 % (w/v) NaOH for 1 hr., then washed with dilute HCl and subsequently with water until free from chlorides. It was then dried. Both samples were reduced to fine powder (100 mesh). In this state the cellulose was more tractable during the fermentation.

The salts in the inorganic medium were the usual 'analytical reagent' grade which, being by no means free from heavy metals, would probably have carried with them sufficient of these constituents to meet the requirements of the micro-organisms. However, to ensure that the course of digestion would not be influenced by the want of these minor elements which are present always in the normal fodder of ruminants, Fe, Cu, Zn and Co were added to the medium as solutions of the chlorides in sufficient quantity to provide 2 mg. Fe, 1 mg. Cu, 2 mg. Zn, and 0·5 mg. Co/l. of the inorganic medium.

For each digestion, approximately 50 g. (*vac.* dry wt. 100°) of the finely divided cellulose was weighed and suspended in 2·5 l. of a solution containing the following concentrations of inorganic constituents: 0·02 M-$(NH_4)_2SO_4$, 0·02 M-KH_2PO_4, 0·01 M-$MgSO_4$, 0·005 M-$CaCl_2$, together with the traces of heavy metals mentioned above.

The micro-organisms which were used as an inoculum were separated mechanically from the rumen contents of sheep which for some days had been fed on chaffed cereal hay. At slaughter, the paunch was tied off and the contents (3–5 l.) removed and strained through a sieve of fine gossamer while being vigorously stirred; this and all subsequent operations were conducted under an atmosphere of CO_2. The liquid portion was freed further from gross contaminating particles by gentle centrifuging. The micro-organisms in the supernatant liquid were then separated in a Sharples centrifuge. The residual sludge was removed from the cylinder, emulsified in 0·2 M-phosphate buffer,

pH 5·5, and made up to 50 ml. The CO_2 was swept out with pure N_2. Samples for cellulose and carbon determinations were withdrawn and the remainder (which contained between 5 and 8 g. of wet sludge) was transferred without delay to the digestion vessel, already prepared to receive it. The material consisted largely of bacteria, the main contaminants being small fragments of lignin, lignocellulose and some of the micro-fauna which normally inhabit the rumen.

The oxidation-reduction (O/R) potential of the medium during fermentation. During active digestion the potential set up on the indifferent (Pt) electrode immersed in the medium indicated an E_h at pH 7·0 poised in the vicinity of −0·380 V. The observed potentials fluctuated, sometimes rapidly, over a relatively wide range (approximately ±30 mV.). The system responsible seemed too delicately balanced to suggest even an approach to a state of true equilibrium; the more negative potentials suggested rather

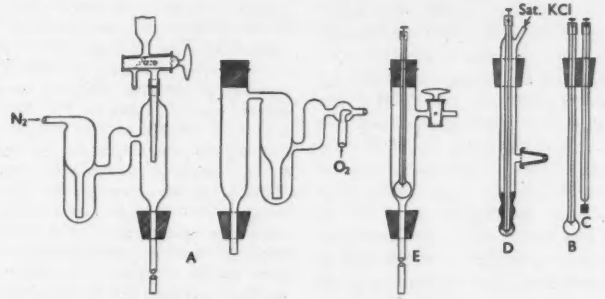

Fig. 2. Subsidiary apparatus introduced through ports in fermentation apparatus: inlet and outlet tubes for gas, etc., and glass, calomel and platinum electrodes. The thermoregulator introduced through the sixth port is not shown.

Procedure of fermentation. After introducing the cellulose, the vessel was closed, the subsidiary apparatus made ready and the contents raised to 40°. The inoculum was run in through one of the ports. The air was swept out with a rapid stream of pure N_2 passed through the inlet tube beneath the surface while the mash was being stirred rapidly. The inlet tube was then withdrawn above the surface, the flow of gas reduced to about 300 ml./hr. and the efferent stream, after dilution with O_2, diverted to the absorption and combustion chain. Standardized 0·5 M-Na_2CO_3 was added drop by drop from the burette, with gentle stirring, until the reaction of the contents was pH 6·8. Stirring was then stopped. Active digestion usually began within an hour and became increasingly rapid. The rate was judged by the change in reaction of the contents as indicated by the glass electrode. The reaction was adjusted at frequent intervals (10–20 min.) by introducing standard Na_2CO_3 from the burette; it was kept by this means between pH 6·8 and 5·5 throughout the course of each fermentation. The stirrer was used only when adjusting the reaction, and then at a rate sufficient merely to impart a gentle swirl to the medium. Rapid stirring was found to reduce the rate of fermentation.

In this way the *milieu* of the rumen was imitated. The conditions were at least very similar to those which prevail normally. The inoculum was relatively massive, and there was a minimum of selection of micro-organisms by the mechanical processes employed to separate them from the normal rumen contents; the reaction of the medium was controlled; the conditions were anaerobic and the CO_2 tension high; and the duration of the fermentation was relatively short. It might reasonably be expected that the course of dissimilation of the cellulose under these conditions would be close to, if not identical with, that taken during normal digestion in the paunch of ruminants.

The results of four successful digestions are reported here.

the production of evanescent, highly reducing intermediates, possibly of hydrogen itself. Active fermentation of cellulose was invariably associated with such low potentials; from observations, however, it was not possible to conclude whether they represented an essential condition for the function of the organisms or whether they were merely the result of the formation of cellulose dissimilation products.

RESULTS

The products of the fermentations in vitro

Gaseous products: CO_2 *and* CH_4. Throughout the course of each experimental digestion the atmosphere above the fermenting mass was swept with pure N_2, continuously, but sufficiently slowly to ensure that the CO_2 tension was not reduced to low levels. The efferent stream, after it had been diluted with pure O_2 and freed quantitatively from H_2O and CO_2, was led through a silica tube packed with CuO and maintained at approximately 700°, and thence through anhydrous $Mg(ClO_4)_2$ and soda-asbestos absorbers to collect the H_2O and CO_2 arising from the combustion. At the completion of a digestion period the introduction of alkali was stopped, the mass was allowed to become acid (pH 5·8–6·0) and then 5 ml. H_3PO_4 (sp.gr. 1·6) were delivered through the inlet port. The N_2 delivery tube was pushed well down beneath the surface, the stream of gas increased, and the mass agitated vigorously with the mechanical stirrer. This procedure was not prolonged further than would ensure removal of the fermentation gases without washing out any appreciable quantity of the more volatile products of digestion.

Methane was the only combustible gas evolved under these conditions of fermentation. Within the very small error of the estimation there was no evidence that the gas evolved contained any free

hydrogen: the ratios of CO_2 and H_2O arising after combustion invariably indicated a carbon/hydrogen ratio of 1 : 4 atoms.

The formation of methane from hydrogen has been recognized since Söhngen (1905) demonstrated the reduction of CO_2 by H_2 with enrichment cultures from soil. Its formation as a product of the CO_2 oxidation of ethanol by *Methanobacterium omelianskii* (Barker, 1936*a*) or of simple fatty acids by *Methanococcus mazei* and by *Methanobacterium söhngenii* (cf. Barker, 1936*b*) are well established reactions. Its production from any compound containing a carbon chain, by an organism isolated by Stephenson & Stickland (1933), reveals further the variety of possible sources of methane in anaerobic fermentations with mixed cultures. The fact that free hydrogen is evolved during the dissimilation of cellulose by 'enrichment' and by 'pure' cultures, or by mixed cultures under conditions which favour thermophilic organisms but in which methane producers could not function, suggests that the bulk of the methane evolved from the rumen may be formed from hydrogen during primary fermentation of complex polysaccharides. Decomposition of other products would be a subsidiary source of methane. Both types of reaction almost certainly occur.

There is thus no reason to expect an inflexible relationship between the amount of methane produced, the amount of substrate fermented, and the CO_2 evolved in the process.

The ratios $(CO_2)/(CH_4)$ in the four fermentations under discussion varied between 3·0 and 3·8 (see Table 2). As the estimation of these constituents was precise, this variation may be attributed to differences in the course of the activity of the mixed cultures. The changes in the ratios do not correlate simply with similar changes in the ratios of the volatile fatty acids, nor with the degree of exhaustion of the substrate. The differences might thus have been due in these experiments to changes in the nature of the original inoculum. The partial pressure of CO_2 above the fermenting mass was not rigidly controlled and may at times have deviated from that which normally prevails in the rumen during active digestion. The fact that free hydrogen was not evolved indicates, however, that at least the CO_2 oxidation of hydrogen was not at any time limited by the CO_2 tension. Between 6·2 and 8·1% of the combustible energy of the cellulose digested was contained in the methane evolved.

The main products: fatty acids. The major part of the undissolved cellulose was collected on a sintered glass funnel. The organisms and fine particles of cellulose which passed the filter were then spun off in a Sharples centrifuge and the residue washed. The insoluble fractions were combined and the cellulose content determined by the method of Norman & Jenkins (1933).

The clarified filtrate and washings were adjusted with NaOH to pH 8·0 and evaporated to about 250 ml. in a still, to which was attached an efficient condenser and a close-fitting receiver immersed in a freezing mixture. Steam was then admitted to the flask and the distillation continued until an additional litre of distillate had collected in the cold receiver. The distillate (D_1) was preserved for the estimation of volatile alcohols, aldehydes, ketones, etc. The residue in the flask was concentrated to approximately 250 ml., acidified with H_3PO_4 to pH 3·0 and then steam distilled without increasing its volume, until approximately 5 l. of distillate (D_2) had collected. The residue (R_1) was preserved for the estimation of succinic acid and the semi-volatile lactic and pyruvic acids. The distillate (D_2) was returned to the still, rendered alkaline, and the volume reduced again to approximately 250 ml. The distillate (D_3) from this, after applying qualitative tests for alcohols, ketones, etc. was discarded. The residue in the flask was acidified again to pH 3·0, and steam distilled until 5 l. of distillate (D_4) had been collected. The residue from this (R_2) was combined with (R_1) and treated as described in the section on intermediates. The distillate (D_4) was exactly neutralized with a known quantity of $Ba(OH)_2$, computed from careful titration of a sample, and then cautiously reduced to dryness and dried finally in a vacuum desiccator. From the weight of the Ba salts and the Ba content the mean combining capacity of the mixed salts was computed. This provided a useful means of evaluating the overall accuracy of the subsequent analyses.

The Ba salts were then taken up in water, decomposed with the calculated quantity of H_2SO_4, and the mixed fatty acids extracted with pure ether in a continuous extraction apparatus. After removal of the ether they were fractionated in an apparatus designed specially for quantitative separation of mixtures of this sort. The column employed was an unpacked spiral glass tube, approximately 10 m. in length, sealed together with a totally enclosed copper reflector into a glass vacuum jacket, the whole being encircled externally with an electrically heated casing. This latter and the external insulation allowed precise adjustment of the thermal gradient. The rate of entry of vapour into the column was determined by a thermoregulator situated at its head, and means were provided for controlling the quantity of liquid taken off. When the apparatus was adjusted critically a sharp separation of the constituents was ensured with a minimum of retention in the fractionating column. The efficiency of the column was equivalent to 24 theoretical plates.

The following fractions were collected: F_1, up to 101°, which contained water and formic acid; F_2, from 101 to 119°, which contained the acetic acid; F_3, from 119 to 142°, which contained the propionic acid; F_4, from 142 to 164°, which contained butyric acid. Transition between the fractions was abrupt. Formic acid was determined in F_1 by reduction of HgO (Osburn, Wood & Werkman, 1933) and butyric acid in F_4 and in the residues by oxidation to acetaldehyde (Klinc, 1934). The composition of fractions F_3 and F_4 was established further by the refractive index and by the combining capacity and the composition of the anhydrous barium salts. The presence of higher homologues in the residuum was doubtful: if they did occur they were in very small quantities.

The results from complete analyses of the products of four digestions are set out in Table 1. The

minor products were not determined in digest 1, but in subsequent experiments these were sought for and estimated quantitatively. They were unimportant in the overall balance sheets of the transactions involved, and the estimations added but little to the understanding of the intermediary stages of the dissimilation.

Elsden (1945), employing partition chromatographic means (Martin & Synge, 1941; Smith, 1942; Ramsey & Patterson, 1945; Elsden, 1946) for the separation of the fatty acids, found in the rumen contents a preponderance of acetic over propionic acid, but he recognized the fact that propionic acid predominated over acetic acid when carbohydrates were fermented under anaerobic conditions in inorganic media inoculated with liquor withdrawn from the paunch. The findings from the *in vitro* fermentations under discussion here are in substantial agreement except for the wide variability of the acetic/propionic ratios found by Elsden. There is, however, no real reason to conclude that the normal course of carbohydrate dissimilation in the rumen varies to the extent implied by Elsden's observations. He made little serious attempt to duplicate the *milieu* of the rumen; the inocula employed were small and the duration of the fermentations prolonged. The possibility that the course of the dissimilation might be altered under such circumstances was greater than when the conditions of the rumen were imitated and the environment controlled throughout.

Table 1. *The products of fermentation of cellulose*

(mmol. = millimols/'mol' (162 g.) of cellulose digested.)

Digest	Formic acid	Acetic acid	Propionic acid	Butyric acid	Methane	CO_2	Cellulose digested (g.)	Duration of digestion (hr.)
1	0·08 g. 12 mmol.	5·3 g. 600 mmol.	11·6 g. 1060 mmol.	0·27 g. 21 mmol.	0·6 g. 250 mmol.	5·4 g. 830 mmol.	24	26
2	0·17 g. 34 mmol.	4·0 g. 610 mmol.	8·5 g. 1050 mmol.	0·3 g. 31 mmol.	0·46 g. 260 mmol.	3·9 g. 810 mmol.	17·8	24
3	0·11 g. 11 mmol.	7·8 g. 620 mmol.	14·4 g. 925 mmol.	1·68 g. 91 mmol.	0·66 g. 195 mmol.	6·9 g. 750 mmol.	34	36
4	0·21 g. 44 mmol.	3·1 g. 500 mmol.	6·4 g. 840 mmol.	1·2 g. 130 mmol.	0·39 g. 236 mmol.	3·2 g. 710 mmol.	16·7	48

The most striking of the analytical findings was the proportion of the main products, propionic acid and acetic acid, and their relationship to the cellulose digested. This relative constancy in the four digests could hardly be fortuitous: it indicates a strong tendency for the main course of dissimilation to proceed through similar if not identical channels. Either the populations of micro-organisms which constituted the inocula were of extraordinarily similar composition, or the selective forces of the environment brought about a relatively stable associative growth. It is probable that the latter were mainly responsible. The inocula were separated from the rumen contents of sheep all of which had been fed similarly for a considerable period prior to slaughter. Each population of micro-organisms thus had ample opportunity to become stabilized.

Table 2. *Molecular ratios of main constituents*

Digest	(Propionic)/(Acetic)	$(CO_2)/(CH_4)$
1	1·8	3·3
2	1·7	3·1
3	1·5	3·8
4	1·7	3·0

The ratios of propionic acid and acetic acid produced in these digests (Table 2) were so similar to those found by Van Niel (1928) to result from the fermentation of lactic acid by certain strains of *Bact. acidii propionici*, as to suggest that organisms of the genus *Propionibacterium* were concerned in their production. Formic acid and butyric acid were present in all digests. The amounts were small and their proportions much more variable. It would appear that these are the products of subsidiary and relatively unstable channels of dissimilation.

Intermediate products. In mixed cultures of this sort, the metabolic end products of the organisms responsible for primary fission of the cellulose would be exposed to further dissimilation. In addition, each soluble intermediate would be subject to the potential competitive action of other organisms, always attendant and ready with a wide diversity of enzyme systems to take advantage of suitable substrates. Intermediary products under such circumstances would be more than usually evanescent and the end products themselves to some degree transient, for the extent of accumulation would be a measure of relative, rather than of complete, stability in this environment.

An exhaustive study of the intermediate compounds may thus not greatly illuminate the processes involved in these mixed fermentations. A search was made, however, for products of subsidiary channels of dissimilation, which might accumulate

in sufficient concentration to be of some nutritional importance to the ruminant. The proportions of propionic and acetic acids in digest 1 suggested that the main course of the dissimilation might proceed through lactic acid (see above). For this reason lactic acid was estimated in digests 2, 3 and 4. The following data from digest 2 are typical of the findings.

Reducing sugars were absent. The distillate (D_1) contained 13 mg. acetaldehyde. Residues (R_1) and (R_2) were combined, reduced in volume, then acidified and exhausted with ether by continuous extraction. The small quantity of extractive, after freeing from ether, was taken up in water and the succinic acid, the aldehydes and ketones, and the semi-volatile lactic and pyruvic acids were estimated. Oxidation of a sample by a modification (Friedemann, Cotonio & Shaffer, 1927) of the von Fürth & Charnass (1910) procedure produced a quantity of acetaldehyde which would imply the presence of approximately 30 mg. lactic acid in the whole digest. The total sulphite-combining capacity of the extractive was of an order which would correspond to less than the equivalent of 6 mg. pyruvic acid, and this would be the upper limit of concentration. As more than 70% of this was destroyed on heating with alkali the concentration of pyruvic acid must have been very small. The quantity was insufficient to allow it to be isolated as the 2:5-dinitrophenylosazone. About 7 mg. succinic acid were identified and about half this amount was isolated. A small quantity of an unidentified ketone or aldehyde was present.

It would appear that lactic acid rather than pyruvic acid tended to accumulate in these digests. This, however, is no proof that lactic acid was a more important intermediate than pyruvic acid in the dissimilation: the reverse might well be true. The prevention of the reduction of lactate by the addition of fluoride would have provided useful evidence on this point, but at the time when the experiments were conducted neither Barker & Lipmann (1944) nor Chaix-Audemard (1940) had published their interesting studies. Attempts were made, which however proved abortive, to accumulate intermediate complexes in the digest by applying Neuberg's *Abfangsverfahren*. Sulphite, when added to an actively fermenting culture apparently acted as a hydrogen acceptor, and altered the course of dissimilation. Hydrogen sulphide was evolved; nitrogenous compounds, which were either introduced in the original inoculum or had been elaborated since, underwent putrefactive changes; in contrast to the light colour and faint sweet odour of the contents of the normal rumen, or of the *in vitro* fermentations under discussion, the mass darkened and stank of indole and mercaptans. Intermediates accumulated by this means would have been of doubtful significance, and so the task of isolating them was set aside.

The minor products identified were not in sufficient quantity to be a nutritionally important energy source for the animal, and they were too small to be considered in the computations of the energy and carbon balance sheets.

The carbon and combustible energy balance sheets

In the four digests that were analyzed in detail, between 84 and 93% of the carbon of the cellulose transformed was recovered in the main products of digestion (see Table 3). There was no evidence which would suggest that either the intermediary complexes or the products from subsidiary channels of dissimilation ever accumulated to an extent which would influence materially the carbon balance sheets. The losses incurred in the chemical isolations were not great, and the small experimental errors entailed in the estimation of the relative proportions of the fatty acids would have comparatively little influence on the assessment of the overall carbon content of these main products. It is not unreasonable, then, to conclude that by far the greater part of the carbon deficit could be accounted for by the carbon bound in the cells of the micro-organisms during their proliferation and growth.

An attempt was made to assess this directly. The main bulk of the undissolved cellulose of digest 3 was separated from the finer particles and micro-organisms by shaking vigorously and straining through fine gossamer. The gross residue was washed in this way several times. The washings were passed through the supercentrifuge after the original filtrate. Cellulose was estimated in the residues which did not pass the sieve and in the sludge from the supercentrifuge by the method of Norman & Jenkins (1933), and the total carbon in the latter was determined by the wet-digestion method of Backlin (1930). The difference between the total carbon content of these residues and the carbon of the cellulose in them was taken as carbon bound in micro-organisms, corrections being applied for the carbon introduced in the original inoculum. Approximately 75% of the carbon deficit in the soluble products was accounted for in this way. It was at best an approximate estimate. Sampling difficulties were encountered, and only a partial recovery of micro-organisms was realized.

The proportion of the carbon of the digested cellulose which was present in the fermentation products was greater when the period of digestion was not prolonged. This suggests that as digestion proceeded, conditions became more favourable for the multiplication of organisms other than those which brought about the primary cleavage of the cellulose; the extent of proliferation of these latter might be expected to bear a more direct relationship to the amount of cellulose digested while this substrate was present in large excess.

The carbon and combustible energy-balance sheets (Tables 3 and 4) indicate that the cellulose fermentation products contained 6% less combustible energy than that of the cellulose fermented. This deficit is an approximate estimate of the free

Table 3. *Carbon contents of the constituents of four digests analyzed in detail*

Digest	Formic acid carbon (g.)	Acetic acid carbon (g.)	Propionic acid carbon (g.)	Butyric acid carbon (g.)	Methane carbon (g.)	Carbon dioxide carbon (g.)	Cellulose digested carbon (g.)	Carbon recovered in products (%)
1	0·02	2·1	5·6	0·15	0·45	1·5	10·7	92
2	0·04	1·6	4·1	0·16	0·34	1·1	7·9	93
3	0·03	3·1	7·0	0·91	0·49	1·9	15·1	89
4	0·06	1·25	3·1	0·65	0·29	0·85	7·4	84

Table 4. *Combustible energy contents of the constituents of four digests analyzed in detail*

Digest	Formic acid (kg.cal.)	Acetic acid (kg.cal.)	Propionic acid (kg.cal.)	Butyric acid (kg.cal.)	Methane (kg.cal.)	Carbon dioxide (kg.cal.)	Cellulose digested (kg.cal.)	% of kg.cal. in products
1	0·1	18·5	57·5	1·6	7·9	—	100	86
2	0·2	14	42	1·8	6·0	—	74	87
3	0·1	27	71	10	8·7	—	140	83
4	0·3	10·8	32	7·1	5·1	—	70	79

energy in the conversion of cellulose to these products, as entropy changes in transformations of this sort are sufficiently small to be within the experimental errors entailed in the chemical estimations on which the computations were based. This energy would be available for the metabolic processes of the micro-organisms. As the relationships between the carbon and combustible-energy contents of cellulose and of the organisms which proliferate during these fermentations are approximately the same,* the amount of energy dissipated as heat in the exothermic metabolic processes of the micro-organisms would be practically identical with the free energy of the dissimilation. The energy balance sheet of the fermentation would thus be as set out in Table 5.

Table 5. *The energy balance sheet of the dissimilation.*

	Combustible energy of cellulose fermented		
Digest	In soluble products and methane (%)	In micro-organisms (%)	Dissipated as heat (%)
1	86	8	6
2	87	7	6
3	83	11	6
4	79	16	5

The fact that the temperature of the rumen contents during active fermentation of hay is above body temperature was pointed out by Krzywanek (1929).

* The combustible energy in bacteria of this type is close to 9·2 kg.cal./g. carbon, the carbon content being about 50 % of their dry weight and the combustible energy between 4·6 and 4·7 kg.cal./g. dry weight (Rubner, 1904). In cellulose it is 9·4 kg.cal./g. carbon, the carbon content being 44·4 % and the combustible energy 4·18 kg.cal./g. The former would thus be approximately 98 % of the latter.

DISCUSSION

Rumen fermentation and the energy metabolism of the ruminant

The associative growth of organisms in the rumen

It seems likely that the course of dissimilation of cellulose in the rumen is determined primarily by the *milieu*, for it is highly probable that any of the vast variety of micro-organisms which inhabit the soil might find their way into the paunch. Those able to flourish anaerobically on the intermediary and end products of the primary fission of complex carbohydrates would form, with the organisms responsible for this first cleavage, a more or less stable population whose composition would be determined by the mutually beneficial relationships between the strains able to take their place in this community.

It might be expected that under the influence of the selective forces of the environment, the process of dissimilation by an associative growth of this sort would tend strongly to assume a pattern, which in many respects would be analogous to the orderly series of dehydrogenations which take place when the simpler carbohydrates are degraded under aerobic conditions in the animal organism.

Viewed in this light, it would not be remarkable if the nature and proportions of the products of fermentation in the rumen were similar, irrespective of the variations in composition of the substrate which obtain under natural feeding conditions. The simple fatty acids represent a relatively refractory stage of carbohydrate dissimilation in the environment of the paunch and, for this reason, accumulate. They are removed from the rumen too rapidly for secondary changes to assume any but an unimportant role. If, however, the *in vitro* digests are left exhausted of their substrate, further anaerobic degradation of these temporary 'end products' takes place. The

final stage would probably be the complete conversion to carbon dioxide and methane found by Hoppe-Seyler (1886) when cellulose was fermented for 4 years with cultures from mud.

The influence of starch and other supplements on the energy transactions in the rumen

If diets containing a high proportion of starch are fed, the rate of proliferation of the microflora in the rumen is limited usually by the nitrogen supply, for, when additional nitrogen is provided as ammonium salts or as simple amides, a large proportion of it is converted to protein through the rapid proliferation of micro-organisms. A considerable part of the combustible energy of the starch may, under these circumstances, escape in part the losses inevitably associated with fermentative degradation. The thermodynamic costs entailed in the production of these organisms are not known, and so the overall effects, which microbial syntheses in the presence of starch have on the supply of energy to the animal, cannot at present be assessed.

When the substrate is composed essentially of the cellulose, cellulosans and polyuronides, etc. of the natural fodder of ruminants, proliferation of the microflora is not influenced so materially by the nitrogen supply, and supplements of simple nitrogenous compounds are utilized poorly if at all by this population. The combustible energy in the bacteria, which pass from the rumen of sheep on this fodder, may be considerably less than the amount estimated from the *in vitro* fermentations discussed above, for *in vivo* the products of fermentation are removed from the rumen and so proliferation at their expense is minimized. In the overall energy economy of an animal on its natural fodder this course in the energy transactions of rumen digestion, although an important adjunct, is a subsidiary one; there is little doubt that the fatty acids arising from the dissimilation of carbohydrates in the paunch provide for the ruminant its main source of energy.

The usefulness of the fermentation products as sources of energy to the animal

The high degree of efficiency, with which the ruminant is able to derive energy from the more complex carbohydrates in its fodder, is offset by the losses associated with the fermentation of starch and of the simpler sugars. In other animals these latter suffer no appreciable loss of combustible energy when they are hydrolyzed by the enzymes of the alimentary canal. They are absorbed as simple sugars before the ingesta pass to the caecum, where micro-organisms ferment in part the more refractory carbohydrate complexes which are unchanged by the digestive enzymes.

The energy represented by the heat dissipated in the metabolic processes of the micro-organisms responsible for the dissimilation in the rumen would be of no benefit to an animal in a warm environment: under cold conditions, however, when the heat radiated by the animal exceeds that evolved by the normal processes of its metabolism, this fraction would spare its equivalent of the combustible energy of nutrients which otherwise would be oxidized in order to maintain body temperature. The combustible energy of that part of the carbohydrates which becomes converted to micro-organisms is apparently well utilized by the animal, for there is ample evidence that micro-organisms which proliferate in the paunch are well digested and are absorbed when they pass to lower levels of the tract. A considerable proportion of this fraction, however, would be represented as protein, and so would be subject to the usual losses associated with protein metabolism.

The products of the rumen fermentation of carbohydrates differ widely in their usefulness to the animal. Methane is expelled and so its combustible energy will be a total loss. Butyric acid might be expected to be utilized efficiently in the fat cycle. The whole of the combustible energy of the propionic acid is probably capable of supporting the processes involved in the maintenance, growth and production of the animal; propionic acid is readily convertible to glucose (Ringer, 1912; Deuel, Butts, Hallman & Cutler, 1935–6) and so would enter the carbohydrate cycle. Acetic acid, however, is by no means so adaptable in metabolism. While there is a great body of evidence which indicates that in the animal it is not convertible to carbohydrate precursors (Ringer & Lusk, 1910; Ringer, 1913; Deuel & Milhorat, 1928; Ponsford & Smedley-MacLean, 1932; Stöhr, 1933; Deuel *et al.* 1935–6; Cross & Holmes, 1937), it is not improbable from more recent evidence that the fat and carbohydrate cycles may be linked through 2-C compounds. The ease and versatility of carbon dioxide fixation, however, renders difficult the assessment of energy costs incurred during the transfer of acetic acid into productive channels. As carbon dioxide labelled with isotopic carbon finds its way into glycogen (Solomon, Vennesland, Klemperer, Buchanan & Hastings, 1941; Buchanan, Hastings & Nesbett, 1942; Vennesland, Solomon, Buchanan & Hastings, 1942) the interpretation of the mechanism, through which the carbon isotope from C^{13}-carboxyl-tagged acetate is transferred to glycogen, is complicated, and as yet there is no general agreement as to the course it takes (Buchanan *et al.* 1943; Lorber, Lifson & Wood, 1945).

If acetic acid is rapidly oxidized and its combustible energy dissipated as heat without entering into the productive channels of metabolism, its combustible energy must be classed with the heat evolved in the exothermic metabolic processes of the

micro-organisms responsible for the dissimilation of carbohydrate in the rumen, for it would serve only to spare other foodstuffs when the animal is in a cold environment. If acetic acid enters the carbohydrate cycle without appreciable loss, its combustible energy should be classed with that of propionic and butyric acids.

In the former case the products of the *in vitro* fermentations of cellulose would have for the animal a productive value between 67 and 72 % of the combustible energy of the cellulose fermented (i.e. 2·8–3·0 kg.cal./g. cellulose); in the latter, if acetic acid follows a course similar to that of propionic acid or butyric acid and enters without loss into either the carbohydrate or fat cycle, the productive value of the fermentation products would be between 85 and 88 % of the combustible energy of the cellulose (i.e. 3·6–3·7 kg.cal./g. cellulose).

The problem of the comparative usefulness of acetic and propionic acids in ruminant nutrition has been investigated through the direct approach of animal calorimetry. Experiments to determine the capacity of acetate and propionate to provide energy, to spare tissue destruction in the fasting sheep and to promote a positive energy balance when added to maintenance rations will be discussed elsewhere.

SUMMARY

1. Under environmental conditions similar to those in the normal rumen, cellulose, when fermented *in vitro* by associative growths of micro-organisms separated from actively fermenting rumen contents, yielded acetic acid, propionic acid, carbon dioxide and methane as main products, together with smaller quantities of formic acid and butyric acid, and traces of acetaldehyde, pyruvic acid and lactic acid.

Propionic acid predominated among the dissimilation products; the molecular ratio of propionic to acetic in four digests ranged from 1·5 to 1·8. The molecular ratios of carbon dioxide to methane ranged from 3·0 to 3·8.

2. Depending on the duration of the fermentation the amount of combustible energy in the micro-organisms which proliferated ranged between 7 and 16 % of that of the cellulose fermented. The cost of production and maintenance of these organisms, estimated from the carbon and energy balance sheets, was approximately 6 % of the combustible energy of the cellulose fermented. As the relationship between the carbon and combustible-energy contents of the micro-organisms and that of the cellulose is approximately the same (9·4 kg.cal./g. C), the energy dissipated as heat during fermentation was close to the energy deficit of the products of fermentation, viz. 6 % of the combustible energy of the cellulose fermented.

3. The energy metabolism of the ruminant is discussed in light of these findings, and it is concluded that the useful energy of the dissimilation products would be between 85 and 88 % of the combustible energy of the cellulose (i.e. between 3·6 and 3·7 kg.cal./g. cellulose) if the acetic acid enters without loss into productive channels of metabolism, and between 67 and 72 % of the combustible energy of the cellulose (i.e. between 2·8 and 3·0 kg.cal./g. cellulose) if the acetic acid is oxidized and wastefully dissipated. These figures would apply to the ruminant in a warm environment; under cold conditions both heat evolved during the dissimilation and the combustible energy of the acetic acid, irrespective of whether it is otherwise useful to the animal, would be available to spare other nutrients which would be oxidized to maintain body temperature.

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REFERENCES

Backlin, E. (1930). *Biochem. Z.* **217**, 482.
Barcroft, J., McAnally, R. A. & Phillipson, A. T. (1944*a*). *J. exp. Biol.* **20**, 132.
Barcroft, J., McAnally, R. A. & Phillipson, A. T. (1944*b*). *Biochem. J.* **38**, ii.
Barcroft, J., McAnally, R. A. & Phillipson, A. T. (1944*c*). *J. exp. Biol.* **20**, 120.
Barker, H. A. (1936*a*). *Arch. Mikrobiol.* **7**, 404.
Barker, H. A. (1936*b*). *Arch. Mikrobiol.* **7**, 420.
Barker, H. A. & Lipmann, F. (1944). *Arch. Biochem.* **4**, 361.
Buchanan, J. M., Hastings, A. B. & Nesbett, F. B. (1942). *J. biol. Chem.* **145**, 715.
Buchanan, J. M., Hastings, A. B. & Nesbett, F. B. (1943). *J. biol. Chem.* **150**, 413.
Chaix-Audemard, P. (1940). *Sur les relations existant entre respiration et fermentation chez propionibacterium pentosaceum.* Inaugural dissertation. University of Lyon.
Colin, G. (1886). *Traité de physiologie comparée des animaux*, 3rd ed. **1**. Paris: J. B. Baillière et fils.
Cross, M. C. A. & Holmes, E. (1937). *Brit. J. exp. Path.* **18**, 370.
Danielli, J. F., Hitchcock, M. W. S., Marshall, R. A. & Phillipson, A. T. (1945). *J. exp. Biol.* **22**, 75.
Deuel, H. J. Jr., Butts, J. S., Hallman, L. F. & Cutler, C. H. (1935–6). *J. biol. Chem.* **112**, 15.
Deuel, H. J. Jr. & Milhorat, A. T. (1928). *J. biol. Chem.* **78**, 299.
Duclaux, E. (1874). *Ann. Chim. (Phys.)*, **2**, 289.

Duclaux, E. (1882). *C.R. Acad. Sci., Paris*, **94**, 976.
Duclaux, E. (1895). *Ann. Inst. Pasteur*, **9**, 265.
Duclaux, E. (1900). *Traité de Microbiologie*, **3**, 394. Paris: Masson et Cie.
Elsden, S. R. (1945). *J. exp. Biol.* **22**, 51.
Elsden, S. R. (1946). *Biochem. J.* **40**, 252.
Friedemann, T. E., Cotonio, M. & Shaffer, P. A. (1927). *J. biol. Chem.* **73**, 335.
Fürth, O. von & Charnass, D. (1910). *Biochem. Z.* **26**, 199.
Gray, F. V. (1947). *J. exp. Biol.* **24**, 1.
Haubner, G. C. (1855). *J. Landw.* **6**, 177.
Henneberg, W. & Stohmann, F. (1860–4). *Beiträge zur Begründung einer rationellen Fütterung der Wiederkäuer*, 1st ed. (1860), pp. 100 and 227; 2nd ed. (1864), p. 342. Brunswick: Schwetschke.
Henneberg, W. & Stohmann, F. (1885). *Z. Biol.* **21**, 613.
Honcamp, F. (1909). *S.B. naturf. Ges. Rostock*, **1**.
Honcamp, F. (1927). *Cellulose Chem.* **8**, 81.
Hoppe-Seyler, F. (1886). *Hoppe-Seyl. Z.* **10**, 401.
Kline, L. (1934). *Biochem. Z.* **273**, 1.
Knieriem, W. von (1885). *Z. Biol.* **21**, 67.
Krzywanek, F. W. (1929). *Pflüg. Arch. ges. Physiol.* **222**, 89.
Lehmann, F. (1889). *J. Landw.* **37**, 251.
Lorber, V., Lifson, N. & Wood, H. G. (1945). *J. biol. Chem.* **161**, 411.
Lusk, G. (1921). *J. biol. Chem.* **49**, 453.
Markoff, J. (1911). *Biochem. Z.* **34**, 211.
Markoff, J. (1913). *Biochem. Z.* **57**, 1.
Marston, H. R. (1939). *Ann. Rev. Biochem.* **8**, 566.
Martin, A. J. P. & Synge, R. L. M. (1941). *Biochem. J.* **35**, 1358.
Milhorat, A. T. & Deuel, H. J. Jr. (1926–7). *Proc. Soc. exp. Biol., N.Y.*, **24**, 667.
Norman, A. G. & Jenkins, S. H. (1933). *Biochem. J.* **27**, 818.
Osburn, O. L., Wood, H. G. & Werkman, C. H. (1933). *Industr. Engng Chem.* (Anal. ed.), **5**, 247.
Ponsford, A. P. & Smedley-MacLean, I. (1932). *Biochem. J.* **26**, 1340.
Ramsey, L. L. & Patterson, W. I. (1945). *J. Ass. off. agric. Chem., Wash.*, **28**, 644, 651.
Ringer, A. I. (1912). *J. biol. Chem.* **12**, 511.
Ringer, A. I. (1913). *J. biol. Chem.* **14**, 43.
Ringer, A. I. & Lusk, G. (1910). *Hoppe-Seyl. Z.* **66**, 106.
Rubner, M. (1904). *Arch. Hyg., Berl.*, **49**, 355.
Scheunert, A. & Trautmann, A. (1921). *Pflüg. Arch. ges. Physiol.* **192**, 33.
Seillière, G. (1910). *C.R. Soc. Biol., Paris*, **68**, 991.
Smith, E. L. (1942). *Biochem. J.* **36**, xxii.
Söhngen, N. L. (1905). *Zbl. Bakt.* (2 Abt.), **15**, 513.
Solomon, A. K., Vennesland, B., Klemperer, F. W., Buchanan, J. M. & Hastings, A. B. (1941). *J. biol. Chem.* **140**, 171.
Stephenson, M. & Stickland, L. H. (1933). *Biochem. J.* **27**, 1517.
Stöhr, R. (1933). *Hoppe-Seyl. Z.* **217**, 141.
Tappeiner, H. (1882). *Ber. dtsch. chem. Ges.* **15**, 999.
Tappeiner, H. (1883). *Ber. dtsch. chem. Ges.* **16**, 1734.
Tappeiner, H. (1884). *Z. Biol.* **20**, 52.
Tappeiner, H. (1888). *Z. Biol.* **24**, 105.
Trautmann, A. (1933). *Arch. Tierernähr. Tierz.* **9**, 178.
Van Niel, C. B. (1928). *The Propionic Acid Bacteria.* Haarlem: N. V. Uitgeverszaak, J. W. Boissevain.
Vennesland, B., Solomon, A. K., Buchanan, J. M. & Hastings, A. B. (1942). *J. biol. Chem.* **142**, 371, 379.
Washburn, L. E. & Brody, S. (1937). *Bull. Mo. agric. Exp. Sta.* 263.
Watson, R. H. (1933). *Aust. J. exp. Biol. med. Sci.* **11**, 197, 253.
Wildt, E. (1874). *J. Landw.* **22**, 1.
Wilsing, H. (1885). *Z. Biol.* **21**, 625.
Zuntz, N. (1879). *Landw. Jb.* **8**, 65.

The Estimation of Suramin in Plasma

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The colorimetric estimation of suramin (Antrypol, Bayer 205) by conversion of the hydrolytic fission products to an azo dye has been described by several investigators. Dangerfield, Gaunt & Wormall (1938) have summarized the earlier work, and have described in detail a method in which the drug is subjected to acid hydrolysis; the fragments are then diazotized and coupled with methyl-α-naphthylamine. Spinks (1948) has used alkaline hydrolysis and *N*-β-sulphatoethyl-*m*-toluidine as a dyestuffs end component. Balaban & King (1927), in their investigation of the affinity of suramin and other complex ureides for cellulose, devised a roughly quantitative method in which the concentration was determined from the turbidity produced by the addition of a basic protein such as clupeine sulphate; for this method the authors claimed a sensitivity of 1 in 100,000.

In this investigation the possibility of a direct, colorimetric estimation by a dye-laking procedure has been explored by examining the behaviour of a range of basic dyes in the presence of the drug. Certain styrylquinoline dyes have been found to exhibit a marked colour change under these conditions, and 2-*p*-dimethylaminostyryl-6-acetamidoquinoline methochloride has been selected as the

most suitable for the estimation. This compound was described by Browning, Cohen, Ellingworth & Gulbranson (1926); it is 'styrylquinoline 24' in their series and will be referred to here as *SQ* 24.

In the presence of excess suramin the deep-red colour of *SQ* 24 is converted to a pale yellow; the change in absorption spectrum is shown in Fig. 1.

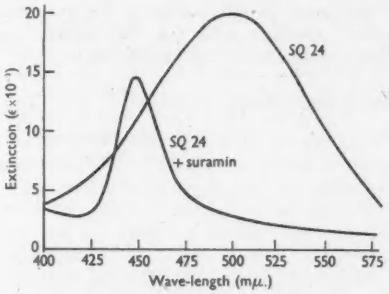


Fig. 1. Absorption spectra of *SQ* 24, with and without added suramin.

SQ 24 has a broad maximum at 495 mμ., and the complex with suramin has a sharp maximum at 450 mμ. The greatest difference between the two curves is to be found at 505 mμ. The complex cannot be extracted by organic solvents, and on standing overnight the yellow aqueous solution deposits a flocculent brown precipitate. If a sufficient concentration of water-miscible organic solvent, such as ethanol or cellosolve, is added to the yellow solution the original red colour of the reagent is regenerated. Plasma proteins do not interfere with the suramin reaction, and the concentration of the drug in plasma may be determined by comparing the optical density of the dye solution, to which has been added diluted plasma (in sufficient 0·9 % NaCl to prevent globulin precipitation), with that of the dye solution without suramin.

METHOD

The analytical procedure described below for plasma is suitable for serum or for aqueous solutions of suramin. The quantities given are for plasma samples containing 0·5–15 mg./100 ml.; for greater or lesser amounts of suramin the ratio of sample to dye solution should be suitably modified.

Reagents required. (*a*) 2-*p*-dimethylaminostyryl-6-acetamidoquinoline methochloride (*SQ* 24) solution; 1 mg./100 ml. distilled water. (*b*) Isotonic sodium chloride (0·9 % NaCl in water).

Apparatus required. (*a*) 6 × ¾ in. test tubes with graduation mark at 10 ml. (*b*) Suitable colorimeter. If none is available, an approximate estimate of the suramin concentration may be made by comparison with a set of freshly prepared standards. In this investigation the Coleman spectrophotometer at a wave length 505 mμ. has been used employing cylindrical cells. Satisfactory results have also been obtained with the Spekker absorptiometer using an Ilford 603 blue-green filter and 1 cm. cells. To avoid adsorption of the dye on the surface of glassware it is important that the tubes and colorimeter cells should be free from surface etching; they should be well cleaned in chromic-sulphuric acid mixture before use.

Procedure

(*a*) Pipette 0·2 ml. plasma into 2 ml. isotonic NaCl solution in a tube graduated at 10 ml. Add 5 ml. *SQ* 24 solution and make up to 10 ml. with distilled water.

(*b*) Pipette 5 ml. *SQ* 24 solution into 2 ml. isotonic NaCl and make up to 10 ml. with distilled water.

(*c*) Pipette 0·2 ml. plasma into 2 ml. isotonic NaCl and make up to 10 ml. with distilled water. The solutions are allowed to stand for a few minutes and the optical densities measured in a suitable colorimeter. The density of the plasma blank (*c*) is subtracted from that of (*a*), and the difference subtracted from the dye blank (*b*). The amount of suramin present in (*a*) may then be determined by applying this difference to a standard curve, constructed from solutions containing known amounts of suramin. Fig. 2 shows standard curves constructed by the above method; curve *A* is for dilutions of suramin in water, while to obtain curve *B* the solutions contained a total concentration of 20 % (v/v) serum. With the amounts of plasma or serum used in the method as described above, the standard curve coincides with the water curve *A*.

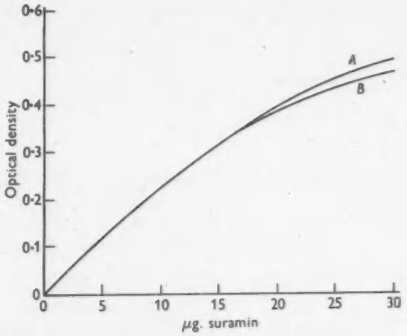


Fig. 2. Standard curve for suramin estimation, *A* in water, *B* in 20 % serum.

DISCUSSION

Comparison with the hydrolysis method

Samples of oxalated plasma, taken at intervals from a rabbit dosed intravenously with 100 mg./kg. of suramin, were subjected to the above analytical procedure and also to the hydrolysis method of Dangerfield *et al.* (1938), modified by the use of the Rose & Bevan (1944) end component *N*-β-sulphatoethyl-*m*-toluidine. The results by the two methods are compared in Table 1.

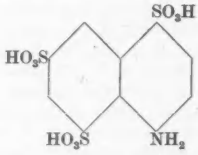
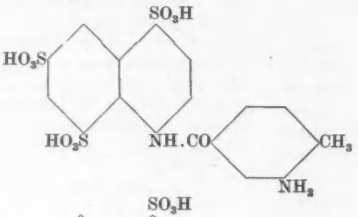
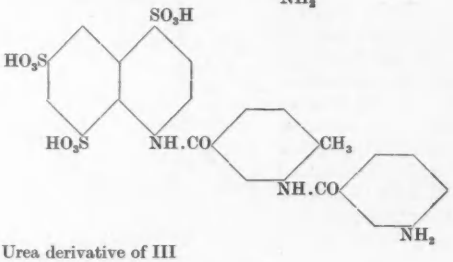
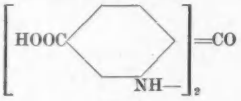
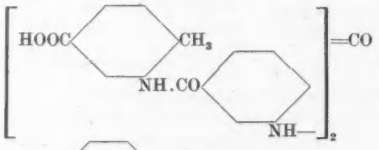
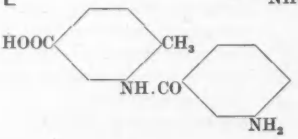
Table 1. *Concentration of suramin in the plasma of an injected rabbit, by dye-laking and hydrolytic methods*

Time after dosing	Plasma suramin (mg./100 ml.) Dye-laking	Hydrolysis
15 min.	71·2	61·6
5 hr.	25·2	21·6
1 day	16·2	15·2
4 days	6·1	5·7
10 days	2·4	2·5
20 days	2·3	3·2

The blank value of the rabbit plasma prior to dosing was 1·3 mg. by the dye-laking method, and 0·96/100 ml. by the hydrolysis method.

Since the dye-laking method necessitates the measurement of a colour difference it might be expected to be rather less precise that the hydrolysis method, particularly with the lower suramin concentrations. The magnitude of this colour difference, however, makes the dye-laking method rather more sensitive; together with the far greater manipulation necessary for the hydrolysis method, this

Table 2. *Suramin 'equivalents' of possible metabolic fission products*

	Formula	Mol. compound equivalent to 1 mol. suramin
I		0·20
II		0·41
III		0·31
IV	Urea derivative of III	0·84
V		0·20
VI		0·24
VII		0

probably makes the precision of the two methods of about the same order. The very much simpler technique of the dye-laking procedure, and the shorter time required, should make the method of particular value if plasma concentrations are required to be estimated in the field with limited laboratory facilities.

Specificity of the dye-laking method

A number of the possible metabolic fission products of suramin have been subjected to the analytical procedure described above and the results are listed in Table 2.

These results indicate that the dye-laking reaction occurs with those fragments which contain the naphthylaminetrisulphonic acid group and also the bis-urea derivatives. It may be assumed from the results with (VII) that amides and the simpler amino-acids do not give the reaction. It is interesting to note that the order of suramin equivalents of this series of compounds approaches very closely to their order of persistence in the blood stream as found by Spinks (1948).

Table 1 indicates that the results obtained by the two methods are of the same order, the dye-laking method giving slightly the higher figures. This suggests the absence from the plasma of appreciable amounts of simple amino-acids and amides without the naphthylaminetrisulphonic acid group, since these would cause the dye-laking method to give lower apparent plasma concentrations. On the other hand appreciable amounts of naphthylaminetrisulphonic acid would cause the dye-laking method to give a higher apparent concentration, since according to Spinks (1948) the azo dye developed from this compound is weakly coloured. It is possible that the observed higher results with the dye-laking method are due to this cause. Spinks has also shown that persistence for a prolonged period in the blood after intravenous injection is only exhibited by symmetrical polyamides of high molecular weight, which contain naphthylaminepolysulphonic acids as end groups, and that after 10 hr. the amounts of the other products of hydrolysis of suramin in the blood are negligible. Moreover, since all the degradation products examined have no trypanocidal activity, it is very likely that after this period both methods are measuring true suramin concentration and any divergence between them is due to experimental error.

SUMMARY

1. A method for estimating suramin in aqueous solution or in serum and plasma is described. It utilizes the colour change which is observed when suramin is added to a styrylquinoline dye, 2-*p*-dimethylaminostyryl-6-acetamidoquinoline methochloride.

2. The method is of about the same precision as the hydrolysis method of Dangerfield *et al.* (1938) and rather more sensitive; it involves a much simpler and more rapid technique. The two methods yield similar values for the suramin content of the blood of a rabbit injected with suramin.

3. The behaviour of hydrolysis products of suramin has been investigated and the specificity of the method is discussed.

REFERENCES

Balaban, I. E. & King, H. (1927). *J. chem. Soc.* p. 3068.
Browning, C. H., Cohen, J. B., Ellingworth, S. & Gulbransen, R. (1926). *Proc. Roy. Soc.* B, **100**, 293.
Dangerfield, W. G., Gaunt, W. E. & Wormall, A. (1938). *Biochem. J.* **32**, 59.
Rose, F. L. & Bevan, H. G. L. (1944). *Biochem. J.* **38**, 116.
Spinks, A. (1948). *Biochem. J.* **42**, 109.

Sexual Differences in the Storage and Metabolism of Iron

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It is now an accepted fact that hormones and/or vitamins have important parts to play in the exchanges and functions of sodium, chlorine, potassium, calcium and iodine, but their role in the metabolism of iron has not been so conclusively demonstrated or so generally recognized. Bunge (1889) suggested that young female animals put aside reserves of iron for the future needs of reproduction, and that the deviation of iron from the circulation to the storage organs at puberty might explain the high incidence of chlorosis in adolescent girls. In more recent years further evidence has been produced that the metabolism of iron is controlled, to some extent at any rate, by the sex hormones. Women, for instance, tend to have lower concentrations of circulating haemoglobin than do men, apart altogether from the obvious effects which menstruation must have upon their bodily require-

ments (Widdowson & McCance, 1936, 1942; Barer & Fowler, 1936). There is also good evidence that they have less inorganic iron in the serum (Vahlquist, 1941). Pregnancy depletes the iron stores of female rats (Smythe & Miller, 1929; Steenbock, Semb & Van Donk, 1936), and Mitchell (1932) showed that if males and females were given a diet which was deficient in iron the males became anaemic more quickly than the females. Smith & Otis (1937) found that, given the same diet, female rats produced more haemoglobin/g. of available iron than did males, and Steenbock *et al.* (1936) that they accumulated more iron in their livers. Hubbell & Rose (1937) and Smith & Otis (1940) showed that the whole bodies of female rats contained more iron/g. than those of males given similar amounts of iron in their diets; Smith & Otis (1940) further demonstrated that, at a low level of intake, the total amount of iron in the bodies of the females was greater than that in the bodies of the males, despite the fact that the females were smaller animals. Steenbock *et al.* (1936), moreover, showed that castration decreased a female's capacity to store iron, and that injections of follicular hormone into immature females stimulated them to store more iron than their litter mates. This work was reported very briefly and no confirmation or extension of it has been found. It is evident, however, that both in the rat and in man there are quantitative differences in the way in which the two sexes use the iron available to them.

The experiments now to be described were undertaken to confirm some of these findings and to discover to what extent they were true of other species. The work formed part of a more general study of the chemistry of growth and development. A short communication has already been made to the Journées thérapeutiques de Paris (McCance & Widdowson, 1946).

METHODS

Rats, mice, rabbits, guinea pigs, poultry, perch and eels have been used for these experiments. The rats were an inbred black and white strain, and were reared on a stock diet consisting of bread, oats, bran, fish meal, milk and greenstuff. The mice, which were highly inbred, were given similar food, and the rabbits and guinea pigs their usual diets. The mice, rabbits and guinea pigs were obtained from the Pathological Laboratory, Cambridge. The cockerels and pullets were specially reared for the experiment by Chivers and Sons Ltd. They were Light Sussex, and the numbers of females and males were the same. The two sexes received exactly the same diet. The perch and eels were obtained from the Freshwater Biological Station, Wray Castle, Windermere. They had not been reared in captivity.

Total iron was determined after dry ashing by the thioglycollic acid method (McCance, Widdowson & Shackleton, 1936). Inorganic iron in the liver was estimated by the modification of Hill's (1930) method described by Shackleton & McCance (1936).

RESULTS

The effects of age, sex, and reproduction on the concentration of inorganic iron in the livers of rats

Seventy-eight male and 99 female animals were used for these experiments, and the results are shown graphically in Fig. 1. It will be seen that the livers of

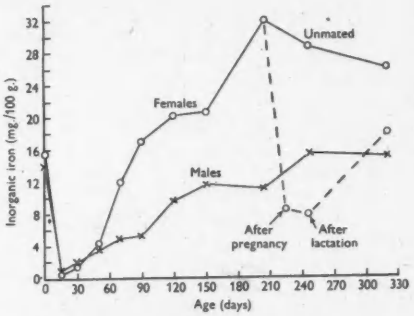

Fig. 1. Concentration of inorganic iron in the livers of male and female rats.

newborn animals contained between 14 and 15 mg. of inorganic iron/100 g. of wet tissue. At the end of a fortnight the inorganic iron had fallen to less than 1 mg./100 g. of liver. This was an expression of the deficiency of iron to which all mammals are liable towards the close of the suckling period. In rats this is not usually accompanied by anaemia, nor does it affect one sex more than another. By the time the young rats were 50 days old the concentration of inorganic iron in the livers of both sexes had risen to about 4 mg./100 g., but with the onset of sexual development the females began to lay down iron in their livers much faster than did the males, and by the time the animals were 90 days old and sexually mature, the female livers contained 17 mg. whereas the male livers contained but 5 mg./100 g. At 200 days the iron in the female livers had reached a level of 34 mg. and in the male livers 11 mg./100 g., so the difference had continued to increase. At this age some of the female animals were mated and at the end of pregnancy the liver iron was found to have fallen to little more than 8 mg./100 g. No increase took place during lactation, but by the time the surviving mothers had reached the age of 320 days, their livers contained 18 mg./100 g. This was a greater concentration of iron than was found in male livers at 320 days, but a lower concentration than was found in the livers of the unmated females.

It is evident that the stores of iron in the livers were drawn upon freely to supply the needs of the foetuses, and the present results can be used to show the extent to which they did so. The liver of an unmated female rat 200 days old contained about 3·8 mg. of inorganic iron. By the end of pregnancy this may be considered to have fallen to 1·1 mg., a difference of 2·7 mg. The bodies of the newborn rats each contained about 0·36 mg. of iron, and, assuming a litter to consist of 8 young animals, this suggests that during pregnancy each mother had to provide 2·8 mg. of iron for her progeny. This is very close to the amount which disappeared from the liver, and suggests that most of the iron in the newborn animals may have come from that source. It would have been interesting to have studied the effects of a second pregnancy, but there were not sufficient animals to enable this to be done.

The effects of castration and sex hormones upon the concentration of inorganic iron in the livers of rats

Eighteen male and 24 female rats were used for these experiments, and they were all killed at the age of 90 days, which is about the time at which rats become sexually mature. The males were divided into three groups. The animals in group 1 were allowed to grow normally, those in group 2 were castrated at the age of 46 days; those in group 3 were also castrated at the same age, but were subsequently treated with subcutaneous injections of stilboestrol; 0·3 mg. of stilboestrol dissolved in arachis oil was injected each day for 3 days, the injection was stopped for 2 days, given for the next 3 days and so on. The females were divided into four groups. The animals in group 1 were allowed to grow normally, and those in group 2 were spayed at the age of 46 days. Those in groups 3 and 4 were also spayed at the same time, but the animals in group 3 were subsequently given stilboestrol by injection, 0·3 mg. a day for 3 days, none for 2 days, the injection continued for 3 days and so on. In this way it was possible to establish a regular oestrous cycle, which was followed by means of vaginal smears. The animals in group 4 were given 0·25 mg. of testosterone each day, also by subcutaneous injection.

The livers of each group were pooled for analysis, and the results are given in Table 1. It will be seen that removing the gonads made the two sexes grow at the same rate, equalized the weights of their livers, and the concentration of inorganic iron in them. The administration of stilboestrol to the castrated males did not increase the percentage of iron in their livers, but it did increase slightly the percentage of iron in the livers of the spayed females. Testosterone did not appear to decrease the percentage of iron in the livers of such animals, or if it did the effect was small. Changes might have been produced by administering larger doses of stilboestrol and testosterone to the male and female castrates respectively, but the present results, taken at their face value, suggest that the male and female hormones may normally act in conjunction with factors present only in bodies of the same sex, for there can be no doubt about the effects of the natural hormones; there are, moreover, the findings of Steenbock *et al.* (1936), to which reference has already been made, that the injection of follicular hormones into immature females increased their capacity to store iron in their livers.

The effects of sexual development on the concentration of inorganic iron in the livers of other animals

Mice. Twelve adult male and 18 unmated female animals were killed. Their livers were removed and those of each sex were pooled for analysis. 1·08 mg. of inorganic iron was found in each 100 g. of male liver and nearly three times as much (3·08 mg./100 g.) in the female organ.

Guinea pigs. Six adult male and 6 adult unmated female animals were killed and the livers were worked up for analysis as already described. The male livers contained 4·9 mg. and the female 4·1 mg. of inorganic iron/100 g.

Table 1. *Effect of castration and of sex hormones on the concentration of iron in the livers of sexually mature rats* (90 *days*)

	Body wt. (g.)	Liver wt. (g.)	Liver as % body wt.	Inorganic Fe in liver: Total/rat (mg.)	Inorganic Fe in liver: mg./100 g.
Males					
No treatment	296	12·7	4·3	0·88	6·9
Castrated	258	9·5	3·7	1·05	11·1
Castrated, and stilboestrol	241	9·4	3·9	0·96	10·2
Females					
No treatment	199	7·3	3·7	1·32	18·2
Spayed	258	10·3	4·0	1·29	12·5
Spayed, and stilboestrol	222	8·8	4·0	1·36	15·5
Spayed, and testosterone	242	9·8	4·0	1·06	10·7

Rabbits. Five male and 6 female animals were killed at the age of 17–25 weeks. The liver of each animal was analyzed separately. Those of the males were found to contain an average of 10·3 mg. and the female livers an average of 8·2 mg. of inorganic iron/100 g.

Eels. Twelve silver males and 11 silver females were caught on their way to the sea; their average weights were 75 and 347 g. respectively. The livers of each sex were pooled for analysis. Those of the males held 28·2 mg. and those of the females 83·5 mg. of inorganic iron/100 g. This figure for inorganic iron in the livers of silver females, although extremely high, is about the value which might have been anticipated from the findings of McCance (1944) for the total iron in the livers of sexually mature females.

Perch. The livers of 11 immature males and of 11 immature females were pooled for analysis. The male fish averaged 24 g. and the females 19 g. in weight. The livers of the former contained 7·5 and of the latter 7·6 mg. of inorganic iron/100 g. Twenty-four male fish and 14 females were caught in May when they were ripe. The male and female fish then weighed 23 and 41 g. respectively. Analysis revealed 9·6 mg. of inorganic iron/100 g. in the male livers and 9·5 mg./100 g. in the female livers. These figures show that the development of the sperm and ova did not take place at the expense of iron stored in the liver.

Poultry. Twelve cockerels and 12 pullets were killed when they were 20 weeks old. Their livers were analyzed separately, and the results are given in Table 2. The average for the cockerels was 8·7 mg.

Table 2. *Concentration of inorganic iron in the livers of cockerels and pullets*

	Liver inorganic Fe (mg./100 g.)	
Bird no.	Cockerels	Pullets
1	8·7	10·5
2	7·0	37·3
3	6·1	20·3
4	11·1	12·8
5	7·0	10·7
6	8·4	20·2
7	9·6	11·5
8	10·3	12·2
9	9·9	10·3
10	13·4	7·0
11	6·0	10·1
12	6·4	13·4
Average	8·7	14·7

and for the pullets 14·7 mg./100 g. The difference was statistically significant ($t = 2{\cdot}49$, $P = 0{\cdot}05$–$0{\cdot}02$). The male spleens contained 4·7 mg. and the female spleens 8·5 mg. of inorganic iron/100 g., so sex appeared to affect the storage of iron in this organ also.

DISCUSSION

The results obtained with rats have confirmed those of previous workers, and there can be no doubt at all that sex has an important influence on the storage of iron in this animal. The same is probably true of mice, but the concentrations of iron are very much lower in this species, so much so that amounts stored can scarcely be of any practical value to either sex. Since, however, sex does not seem to influence the storage of iron in either guinea pigs or rabbits, it is clear that even within the mammalian order these sex differences do not always appear, and they can certainly not be taken to hold for any given species until their presence has been experimentally verified. It will be exceedingly difficult to do this for the human species. While, therefore, these differences can never be ranked among the great biological generalizations, it is stimulating to have found them so easily in each of the three vertebrate orders which have been investigated. It is clear that a great deal more work will have to be done to find out how the sex hormones bring about these effects, and why they do not always do so, for the sex hormones of the rat must be very similar to those of the rabbit or indeed of any other vertebrate. What can the second factor be which enables the sex hormones to exert their effects, or which prevents them doing so? It may be unwise to formulate theories at this stage, but it can do no harm to point out that the female rat has been shown to store vitamin A more readily than the male under identical conditions of feeding and whether carotene or vitamin A is acting as the source of supply. Yet males have higher blood levels (Brenner, Brookes & Roberts, 1942). During periods of depletion, moreover, male rats were found to lose their stores of vitamin A more readily than female rats (Popper & Brenner, 1942). Is this association of iron and vitamin A wholly fortuitous? Few biological discoveries can be fitted into their proper place in the framework of knowledge until some time after they have been made, and they often turn out to have a much more general application than was at first supposed.

SUMMARY

1. With the onset of sexual development, female rats have been found to accumulate iron in their livers at a much greater rate than males.

2. Removal of the gonads from male and female rats made the males store more and the females less iron in their livers, so that the concentrations were equal for the two sexes. The administration of stilboestrol had no effect on the castrated males, but it caused a slight increase in the iron storage of the spayed females.

3. The livers of sexually mature female rats, mice, hens and eels have been shown to have a greater concentration of iron in them than the livers of male animals at a corresponding stage of development.

4. Guinea pigs, rabbits and perch were not found to exhibit these sex differences.

5. Pregnancy rapidly reduced the percentage of iron in the livers of female rats and the reaccumulation was comparatively slow.

The authors wish to thank those who have assisted them to obtain the animals used in this investigation, and Miss E. Wilkinson for operating on the rats. The work was made possible by the help given to the Department by the Medical Research Council.

REFERENCES

Barer, A. P. & Fowler, W. M. (1936). *Amer. J. Obstet. Gynec.* **31**, 979.
Brenner, S., Brookes, M. C. H. & Roberts, L. J. (1942). *J. Nutrit.* **23**, 459.
Bunge, G. (1889). *Hoppe-Seyl. Z.* **13**, 399.
Hill, R. (1930). *Proc. Roy. Soc.* B, **107**, 205.
Hubbell, H. J. & Rose, M. S. (1937). *J. Nutrit.* **13**, 5 (Proc.).
McCance, R. A. (1944). *Biochem. J.* **38**, 474.
McCance, R. A. & Widdowson, E. M. (1946). *Thérapie*, **1**, 308.
McCance, R. A., Widdowson, E. M. & Shackleton, L. R. B. (1936). *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 213.
Mitchell, H. S. (1932). *J. biol. Chem.* **97**, xv.
Popper, H. & Brenner, S. (1942). *J. Nutrit.* **23**, 431.
Shackleton, L. & McCance, R. A. (1936). *Biochem. J.* **30**, 582.
Smith, M. C. & Otis, L. (1937). *J. Nutrit.* **13**, 573.
Smith, M. C. & Otis, L. (1940). *Science*, **91**, 146.
Smythe, C. V. & Miller, R. C. (1929). *J. Nutrit.* **1**, 209.
Steenbock, H., Semb, J. & Van Donk, E. C. (1936). *J. biol. Chem.* **114**, cl.
Vahlquist, B. C. (1941). *Acta Paediat.* **28**, Suppl. v.
Widdowson, E. M. & McCance, R. A. (1936). *J. Hyg., Camb.*, **36**, 13.
Widdowson, E. M. & McCance, R. A. (1942). *Lancet*, **1**, 588.

A Synthesis of *dl*-Hydnocarpic Acid

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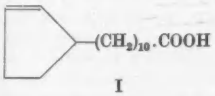
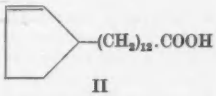
Hydnocarpic acid in its dextro-form is isolated from several oils (e.g. oil of *Hydnocarpus wightiana*) used in the treatment of leprosy. The structure (I)



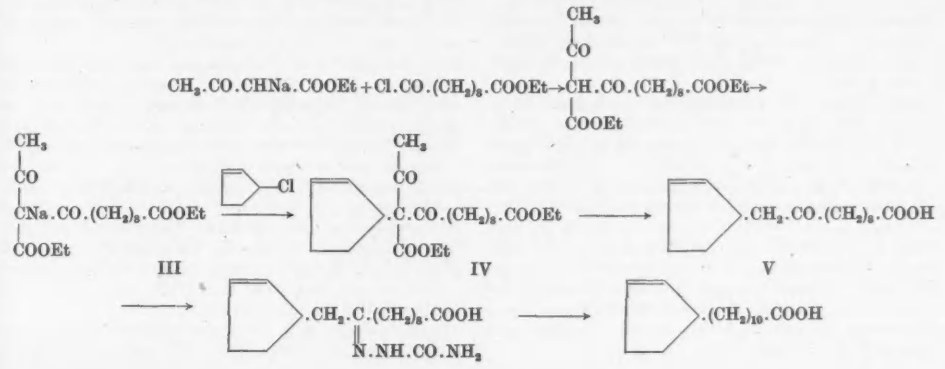
I



II

assigned to hydnocarpic acid rests on the results of permanganate oxidation (Barrowcliff & Power, 1907), the synthesis of dihydrohydnocarpic acid (Noller & Adams, 1926*a*; Bokil & Nargund, 1937), the homologation to and from chaulmoogric acid (II) (Stanley & Adams, 1929; Buu-Hoï, 1944) and a synthesis of *dl*-hydnocarpic acid by a route involving several changes in a *cyclo*pentane ring (Bokil & Nargund, 1941).

The synthesis of *dl*-chaulmoogric acid (II), at the Culion Leper Colony (Perkins & Cruz, 1927*b*) is now a classic, and it was thought worth while to apply the same general method (the Robinson long-chain synthesis, Robinson & Robinson, 1925) to the synthesis of hydnocarpic acid.



The ester-acid chloride of sebacic acid reacts smoothly with ethyl sodioacetoacetate and the product yields a sodio derivative (presumably III). *cyclo*Pent-2-enyl chloride, as a substituted allyl chloride (Noller & Adams, 1926*b*), reacts in the cold with (III) to give the complex (IV); this complex is hydrolyzed, mainly with the loss of the acetyl and the carbethoxy groups, to 10-ketohydnocarpic acid (V). This keto acid is isolated as its semicarbazone which, on heating with sodium ethoxide, yields *dl*-hydnocarpic acid. This specimen of synthetic *dl*-hydnocarpic acid did not depress the melting point of the natural *d*-acid; Hinegardner (1933) found that *dl*-hydnocarpic acid, obtained by racemization of hydnocarpamide, did not depress the melting point of the *d*-acid. Confirmation follows from the preparation of the dihydro derivatives in which there is no asymmetry. The results are summarized in Table 1.

Ethyl 11-(cyclo*pent*-2-*enyl*)-11-*carbethoxy*-10:12-*diketotridecoate*. The cold, toluene solution of the ester obtained above was added slowly to powdered sodium (2·3 g., 1 equiv.) covered with toluene, and the mixture was boiled under reflux for 2 hr., when almost all the metal had reacted. A freshly prepared solution of *cyclo*pent-2-enyl chloride (Perkins & Cruz, 1927*a*; 12·8 g., 1·25 mol.) in toluene (11 ml.) was added: NaCl separated and the mixture became pasty. After refluxing for 1 hr. it had an acid reaction to phenolphthalein. With the aid of water (50 ml.) containing sodium acetate crystals (0·5 g.) the gelatinous mixture was transferred to a 500 ml. flask and steam was passed to destroy or remove *cyclo*pentenyl chloride, *cyclo*pentadiene and toluene. The residual ester was collected with the aid of ether; a sample of it boiled at 180–190°/0·04 mm.; it had $n_D^{20°}$ 1·4511 and gave a red-brown colour with ethanolic $FeCl_3$ solution.

Semicarbazone of 10-*ketohydnocarpic acid*. Hydrolysis of the crude ethyl 11-(*cyclo*pent-2-enyl)-11-carbethoxy-10:12-diketotridecoate (31 g., 1 mol.) in ethanol (50 ml.) was carried out by slowly adding aqueous KOH (24 g. in 30 ml. of water) with vigorous shaking; potassium salts were precipitated and much heat evolved. Water (50 ml.) was added to give a clear brown solution which was boiled for 1 hr. under reflux: a test portion was then miscible with water. Steam was passed through the solution to remove ethanol and *cyclo*pentenylacetone.

Acidification of the alkaline residue in the distilling flask with conc. HCl (50 ml.) precipitated a brown mixture of solid acids (18 g., dry) from which, by rapid distillation at 0·5 mm. pressure, 15 g. of colourless waxy solid, m.p. 85–102°, were obtained. This solid was a mixture mainly of sebacic and 12-ketohydnocarpic acids which could not easily be separated by crystallization. The mixture of acids (15·3 g.) in ethanol (50 ml.) was added to a solution of semicarbazide hydrochloride (5 g.) and hydrated sodium acetate (6·5 g.) in water (50 ml.). After the resulting mixture had been warmed for 15 min. on the water bath it was allowed to stand overnight: 11·0 g. of colourless prisms, m.p. 125–127°, were deposited. (Yield 28 %, calculated on the ethyl 11-(*cyclo*pent-2-enyl)-11-carbethoxy-10:12-diketotridecoate.) Recrystallized from ethanol and then from *n*-propyl alcohol-benzene the substance melted at 135·5–136·5°. (Found: C, 63·0; H, 9·1; N, 12·8 %; equivalent, by titration, 318. $C_{17}H_{29}O_3N_3$ requires C, 63·1; H, 9·0; N, 13·0 %; equivalent, 323.)

dl-Hydnocarpic acid. The purified semicarbazone (3·7 g., 1 mol.) was heated with sodium ethoxide (from 0·77 g. Na,

Table 1. *Melting points of natural and synthetic acids and derivatives*

	Found		
	Synthetic *dl*	Natural *d*	References
Hydnocarpic acid	59– 59·5°	59·5– 60°	*dl* 58·5–59° (Hinegardner, 1933) *dl* 58–59° (Synthetic; Bokil & Nargund, 1941) *d* 59–60° (Power & Barrowcliff, 1905) *d* 60·5° (Cole & Cardoso, 1937)
Hydnocarpamide	108·5–109°	109–109·5°	*dl* 109–110° (Synthetic; Bokil & Nargund, 1941) *d* 111–112° (Hinegardner, 1933)
Dihydrohydnocarpic acid	62·5– 63°	63°	63–63·5° (Noller & Adams, 1926*a*) 63° (Wrenshall & Dean, 1924)
Dihydrohydnocarpamide	114·5–115·5°	114·5–115·5°	114–115° (Noller & Adams, 1926*a*)

EXPERIMENTAL

9-*Carbethoxynonoyl chloride*. Ethyl hydrogen sebacate, prepared from diethyl sebacate by the procedure of Grün & Wirth (1922) and crystallized from light petroleum (b.p. 60–80°) melted at 36·5°. (Found: C, 62·3; H, 9·6 %. Calc. for $C_{12}H_{22}O_4$: C, 62·6; H, 9·6 %.) Refluxing for 4 hr. with excess of thionyl chloride gave carbethoxynonoyl chloride as a viscous oil, b.p. 141–144°/0·14 mm., or 168–170°/14 mm. (yield 80 %). By the action of ice-cold aqueous ammonia 9-*carbethoxynonamide*, m.p. 68·5–69°, was prepared. (Found: C, 63·2; H, 9·9; N, 6·7 %. $C_{12}H_{23}O_3N$ requires C, 63·0; H, 10·0; N, 6·2 %.)

Ethyl 10:12-*diketo*-11-*carbethoxytridecoate*. Sodium (2·3 g., 1 equiv.) was powdered under xylene and the powder washed with dry toluene. Dry toluene (70 ml.) was added and then, with cooling, 13 g. (1 mol.) of redistilled ethyl acetoacetate; the mixture was allowed to stand with occasional shaking until all the sodium had reacted (1 hr.).

Freshly prepared 9-carbethoxynonoyl chloride (24·8 g., 1 mol.) in toluene (20 ml.) was then added slowly with shaking and after half an hour the mixture was boiled under reflux until it became neutral to litmus (30 min.). The resulting ethyl 10:12-diketo-11-carbethoxytridecoate was not isolated: its sodio derivative reacted smoothly with *cyclo*pent-2-enyl chloride.

3 equiv.) in ethanol (9 ml.) at 200° for 21 hr. After acidification the product yielded to ether 2·2 g. (76 % yield) of yellow solid. Rapid distillation at 0·1 mm. followed by crystallization from light petroleum-ethyl acetate gave pearly plates of m.p. 59–59·5°. Hinegardner (1933) gave m.p. 58·5–59° for the racemic acid. (Found: C, 76·4; H, 11·1 %; equivalent by titration, 248. Calc. for $C_{16}H_{28}O_2$: C, 76·2; H, 11·1; equivalent, 252.) The amide prepared by the usual method melted at 108·5–109°.

Dihydrohydnocarpic acid (*synthetic*). The *dl*-hydnocarpic acid (0·3 g.) in ethanol (10 ml.) was reduced with hydrogen at 1 atmos. pressure in presence of Raney nickel. In 50 min. 28 ml. of hydrogen (at 756 mm. and 20°) were absorbed (theory, 27·5 ml.) and the product (0·25 g.) melted at 59–60°. Crystallization from light petroleum-ethyl acetate gave pearly plates of m.p. 62·5–63°. (Found: C, 75·5; H, 11·9 %. Calc. for $C_{16}H_{30}O_2$: C, 75·6; H, 11·8 %.) The amide, obtained in the usual way and crystallized from ethanol, melted at 114·5–115·5°. (Found: C, 75·9; H, 12·2 %. Calc. for $C_{16}H_{31}ON$: C, 75·9; H, 12·3 %.)

d-Hydnocarpic acid (*from Hydnocarpus oil*). Oil of *Hydnocarpus wightiana* (40 g.) was boiled for 5 hr. under reflux with ethanolic KOH solution (128 ml. of 2·5 N). Water (50 ml.) was added and, after heating had been continued for a further 10 hr., acidification gave a yellowish, waxy mixture of acids (36 g.). Esterification of these acids by boiling for 21 hr. with ethanol (56 ml.) and H_2SO_4 (3 ml.) gave on cooling a dark layer of ethyl hydnocarpate and ethyl chaulmoograte, sparingly soluble in ethanol (Buu-Hoï, Cagniant & Janicaud, 1941). The ester layer was diluted with ether, washed with water, Na_2CO_3 solution, with water and then dried: it yielded 31 g. of mixed ethyl esters. Repeated fractional distillation of these esters with the aid of a Vigreux column gave 6·1 g. of ethyl hydnocarpate (b.p. 164–165°/0·6 mm., $n_D^{20°}$ 1·4583). (Found: C, 77·0; H, 11·5 %. Calc. for $C_{18}H_{32}O_2$: C, 77·1; H, 11·4 %.) The ethyl chaulmoograte fraction boiled at 172–174°/0·6 mm., $n_D^{20°}$ 1·4600. Hydrolysis of the ethyl hydnocarpate by refluxing with ethanolic KOH (10 % (w/v) KOH, 10 % (v/v) water) for 4·5 hr. gave 5·5 g. of crude acid, m.p. 51–54°. Recrystallization from light petroleum-ethyl acetate gave colourless leaflets, m.p. 59·5–60° alone or when mixed with the specimen of synthetic *dl*-acid. The amide, crystallized from ethanol, melted at 109–109·5° and did not depress the m.p. of a specimen of the synthetic *dl*-amide (m.p. 108·5–109°).

Dihydrohydnocarpic acid (*from the d-acid*). Pure *d*-hydnocarpic acid (0·45 g.) dissolved in ethanol absorbed 43 ml. of hydrogen (theory 41 ml.) at 21° and 756 mm. in presence of Raney nickel: 0·44 g. of acid, m.p. 60·5–62° was obtained. Twice crystallized from light petroleum-ethyl acetate it melted at 63° alone or when mixed with the dihydro acid from synthetic *dl*-hydnocarpic acid.

The amide crystallized from ethanol in long prisms of m.p. 114·5–115·5° (alone or when mixed with the amide from synthetic dihydrohydnocarpic acid. (Found: N, 5·8 %. Calc. for $C_{16}H_{31}ON$: N, 5·5 %.)

SUMMARY

1. *dl*-Hydnocarpic acid has been synthesized from *cyclo*pent-2-enyl chloride by the Robinson long-chain synthesis (Robinson & Robinson, 1925), a method similar to that used by Perkins & Cruz (1927*b*) for chaulmoogric acid.

2. The accepted structure of hydnocarpic acid is confirmed.

We are indebted to Dr J. L. Simonsen, F.R.S., Director of Research, Colonial Products Research Council, Imperial Institute, for a supply of the oil of *Hydnocarpus wightiana*; and to Mr T. M. Sharp, of the Wellcome Research Institution, for a specimen of *d*-hydnocarpic acid.

REFERENCES

Barrowcliff, M. & Power, F. B. (1907). *J. chem. Soc.* **91**, 557.

Bokil, K. V. & Nargund, K. S. (1937). *J. Univ. Bombay*, **6**, I, 93.

Bokil, K. V. & Nargund, K. S. (1941). *Proc. Indian Acad. Sci.* **13** A, 233.

Buu-Hoï, Ng. Ph. (1944). *Ann. Chim.* (*Phys.*), **19**, 446.

Buu-Hoï, Ng. Ph., Cagniant, P. & Janicaud, J. (1941). *C.R. Acad. Sci., Paris*, **212**, 577.

Cole, H. I. & Cardoso, H. (1937). *J. Amer. chem. Soc.* **59**, 963.

Grün, A. & Wirth, T. (1922). *Ber. dtsch. chem. Ges.* **55**, 2206.

Hinegardner, W. S. (1933). *J. Amer. chem. Soc.* **55**, 2831.

Noller, C. R. & Adams, R. (1926*a*). *J. Amer. chem. Soc.* **48**, 1080.

Noller, C. R. & Adams, R. (1926*b*). *J. Amer. chem. Soc.* **48**, 2444.

Perkins, G. A. & Cruz, A. O. (1927*a*). *J. Amer. chem. Soc.* **49**, 517.

Perkins, G. A. & Cruz, A. O. (1927*b*). *J. Amer. chem. Soc.* **49**, 1070.

Power, F. B. & Barrowcliff, M. (1905). *J. chem. Soc.* **87**, 884.

Robinson, G. M. & Robinson, R. (1925). *J. chem. Soc.* **127**, 175.

Stanley, W. M. & Adams, R. (1929). *J. Amer. chem. Soc.* **51**, 1515.

Wrenshall, R. & Dean, A. L. (1924). *Publ. Hlth Bull., Wash.*, **141**, 24.

The Absorption of Ammonia from the Rumen of the Sheep

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The peculiar anatomical arrangement of the digestive tract of the ruminant presents special problems in the elucidation of the processes of digestion in these species. The food is received, after swallowing, into the capacious rumen and reticulum where extensive fermentative changes are effected by the abundant micro-organisms which inhabit these organs. This unique arrangement confers on the ruminants an efficient mechanism for the digestion of cellulose and other polysaccharides of their exclusively herbaceous diet. The favourable conditions maintained in the rumen and reticulum for the growth of micro-organisms result in extensive conversion of the nitrogen of the animal's food to the use of the micro-organisms for their own growth. That ammonia should appear in the ruminal contents is not surprising in view of the fact that ammonia is the chief nitrogenous end product in the breakdown of proteins by bacteria, while at the same time ammonia is the chief source of nitrogen in the nutrition of many bacteria (Stephenson, 1939).

Schwarz & Steinlechner (1925) were unable to detect ammonia in the rumen contents of cattle, but their observation is at variance with that of all subsequent workers, e.g. Mangold & Schmitt-Kramer (1927); Harris, Work & Henke (1943); Wegner, Booth, Bohstedt & Hart (1941); Lenkeit & Becker (1938). Interest in the non-protein nitrogen fractions in the rumen contents has chiefly been directed to the phenomenon of the utilization by ruminants of urea and amides as a source of protein through the mediation of the micro-organisms of the fore stomachs; recent reviews of the extensive literature on this subject have been given by Smith (1945) and McClymont (1946). That urea is rapidly converted to ammonia by microbial activity in the rumen was demonstrated by Lenkeit & Becker (1938) and confirmed by later workers. However, it appears to have been overlooked that the saliva in those species studied contains urea in concentrations proportional to that in the blood (Hench & Aldrich, 1922, 1923; Schmitz, 1923; Komarov & Stavraky, 1940). Observations recorded in this paper show that urea occurs in the saliva of the sheep, and in view of the fact that the sheep secretes very large volumes of saliva (Scheunert & Trautmann, 1921; Scheunert & Krzywanek, 1930) this source of urea, and hence of ammonia, in the rumen contents assumes some significance.

Little attention has previously been paid to the role of ammonia in the digestion of natural foodstuffs in the rumen; some representative data on the changes in the concentration of ammonia in the rumen contents are presented in this paper. Ammonia in the ruminal contents appears to derive chiefly from the deamination of food proteins and from urea secreted in the saliva. Ammonia could disappear from the rumen liquor by its utilization by bacteria for their growth, by passage in the ruminal ingesta from the rumen to the abomasum, or by direct absorption.

Barcroft, McAnally & Phillipson (1944) demonstrated that volatile acids (acetic, propionic and butyric) are absorbed from the rumen. Further, it has long been known that ammonia is absorbed from the intestinal tract of monogastric animals (Folin & Denis, 1912; Parnas & Klisiecki, 1926; Koprowski & Uninski, 1939). These facts suggested that ammonia might be absorbed from the rumen of the sheep; the experiments reported in this paper demonstrate that this is indeed the case.

METHODS

Ammonia in blood was estimated by the method of Conway & Cooke (1939). For the collection of blood from the visceral veins, the technique developed by Barcroft *et al.* (1944) was used. The experimental animal was anaesthetized with nembutal (pentobarbital), the abdomen was opened and the visceral veins exposed. Blood was withdrawn into a syringe, with as little interference to blood flow as possible. No technique is as yet available for withdrawing blood from the ruminal veins in the normal, unanaesthetized animal.

Ammonia in rumen liquor was estimated by the method of Conway (1947) using potassium metaborate as the alkaline reagent. Corrections for the slight deamination caused by the alkali were applied. The rumen liquor was prepared by passing through muslin ruminal contents collected through a permanent rumen fistula.

Urea in saliva was estimated by the method of Conway & O'Malley (1942).

RESULTS

Ammonia in blood

Preliminary analyses of the jugular blood of normal sheep confirmed the general conclusion of Conway (1947) that in the blood of the general circulation there is almost no ammonia present. Conway has shown that ammonia is formed in

human blood within a few minutes after collection, and that during absorption of ammonia in the Conway unit gradual deamination occurs. In the experiments quoted below the technique adopted necessitated the passage of some 2–4 min. from the time of insertion of the hypodermic needle into the vein until the blood sample had been delivered into the Conway units (analyses were performed in triplicate or quadruplicate). No data are available for applying corrections for the rate of ammonia formation or rate of deamination by alkali in the blood of sheep. For this reason Conway's corrections for deamination as applied to human blood have been used, but no correction has been applied for ammonia formation during the interval between collection of the blood and its mixture with the potassium carbonate in the Conway unit. Thus no special accuracy can be claimed for the values given for ammonia in blood drawn from the jugular vein or the carotid artery; it can only be concluded that these values are extremely small. In analyses of blood from the ruminal veins, the errors involved are of comparatively little significance.

The results of six experiments are given in Table 1; it was consistently found that the blood of the ruminal veins contained significant amounts of ammonia; the values averaged 1·7 mg. ammonia N/100 ml. blood.

Barcroft *et al.* (1944) had previously observed that the concentration of volatile fatty acids was higher in the anterior ruminal vein than in the posterior ruminal vein; they ascribed this to the fact that the posterior ruminal vein drained an area of the rumen which covered a gas space, which thus, in part, removed the ruminal mucosa from contact with the ingesta. In Exps. 5 and 6 (Table 1), analyses were made of blood from both ruminal veins and in each case the level of ammonia was slightly higher in the anterior ruminal vein.

The observations made in Exp. 2 confirm the findings of Folin & Denis (1912) and of Parnas & Klisiecki (1926) that ammonia is also absorbed from other regions of the gastro-intestinal tract.

In order to establish that the ammonia found in the ruminal veins is in fact derived from the ruminal ingesta, two experiments were performed. Two sheep were fasted for 24 hr. and then the contents of the rumen and reticulum were removed through a rumen fistula, and the rumen was washed out as thoroughly as possible with warm water. Under nembutal anaesthesia the oesophagus was ligated, the fluid again removed from the rumen and replaced with warm water, and the posterior ruminal vein exposed by laparotomy. Samples of blood from the ruminal vein were taken for estimation of ammonia. The fluid was then removed from the rumen and replaced with 4 l. of a warm solution of ammonium acetate. After an interval of 30 min., blood samples were again taken from the posterior ruminal vein and from the carotid artery. The results of the two experiments are set out in Table 2.

It has been found difficult to wash the rumen quite free of ingesta, and this explains why some ammonia remains in the rumen fluid after washing, and why the blood draining the washed rumen still contains more ammonia than the blood in the general circulation. The marked rise in the level of ammonia in the ruminal blood, after the rumen has been filled with a dilute solution of ammonium acetate, leaves no doubt that ammonia can be absorbed from the rumen.

Table 1. *Concentration of ammonia in the blood from different vessels in sheep*

Vein / Exps.	1	2	3	4	5	6
	Ammonia N (mg./100 ml. blood)					
Jugular	0·0	0·0	0·1	—	—	—
Carotid (artery)	—	—	—	0·07	0·02	0·06
Posterior ruminal	1·9	1·9	1·5	1·4	2·2	1·3
Anterior ruminal	—	—	—	—	2·4	1·6
Abomasal	—	0·1	—	—	—	—
Intestinal	—	0·6	—	—	—	—
Caecal	—	0·8	—	—	—	—

Table 2. *Absorption of ammonia from the rumen of sheep*

Origin of samples	Ammonia N			
	Experiment 7		Experiment 8	
	(mg./100 ml. blood)	(mg./100 ml. ruminal fluid)	(mg./100 ml. blood)	(mg./100 ml. ruminal fluid)
Ruminal fluid, beginning of experiment	—	0·2	—	0·4
Posterior ruminal vein, 1st sample	0·13	—	0·36	—
Ruminal fluid containing ammonium acetate	—	15·4	—	22·6
Posterior ruminal vein, 2nd sample	0·93	—	1·64	—
Carotid artery	0·07	—	0·07	—

Ammonia in the rumen contents

In the course of studies on the digestion of protein in the sheep, numerous analyses of the content of ammonia in the rumen liquor were made; it has been repeatedly observed that ammonia N may constitute a significant fraction of the total N in the rumen liquor. A typical curve showing changes in the concentration of ammonia in the rumen liquor after feeding on pasture are given in Fig. 1;

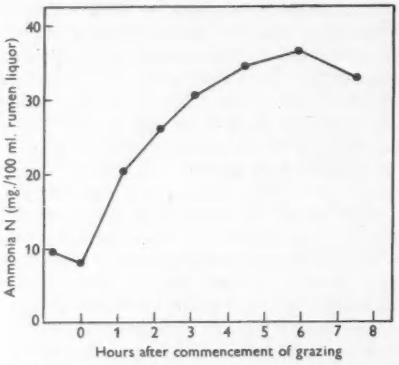

Fig. 1. Change in concentration of ammonia in the ruminal contents of a sheep after grazing at pasture.

the animal was fasted overnight and returned to the pasture after two preliminary samples of rumen liquor had been collected. The figure shows clearly that the concentration of ammonia rose steadily for some hours after feeding on pasture, and attained a maximum value of about 35 mg. ammonia N/100 ml. rumen liquor.

Urea in saliva

Samples of mixed saliva were collected from three sheep on 2 days just prior to feeding. The values obtained for the concentration of urea in the saliva are given in Table 3.

Table 3. *Concentration of urea* N *in the saliva of sheep*

Sheep no.	Urea N on 2 days (mg./100 ml.)
1	8·2, 13·6
2	6·5, 7·4
3	15·0, 10·4

DISCUSSION

The fact that ammonia is absorbed from the rumen raises several interesting points. Since urea is present in the saliva it appears that a N circulation occurs during the normal digestive process; the salivary urea is converted by microbial activity in the rumen into ammonia, which may be absorbed into the venous blood and passed via the portal vein into the liver; here the ammonia will be converted into urea and again become available for secretion in the saliva. The significance of this cycle will clearly be dependent on the relation between the amount of urea N secreted daily in the saliva and the amount of ammonia N absorbed daily, for it seems probable that any nitrogen thus absorbed in excess of that secreted in the saliva would constitute a wastage in metabolism. Data are not yet available to allow accurate assessment of these quantities, but preliminary calculations suggest that ammonia N absorbed may well exceed by significant amounts the urea N secreted in the saliva. Barcroft *et al.* (1944) estimated the rate of flow of blood from the rumen and reticulum as 225 ± 65 ml./min. (This figure has been recalculated from the data of Barcroft *et al.* (1944) as the actual values for *total* blood flow from the rumen and reticulum are not quoted in their paper.) If then the efferent blood contains 1·5 mg. ammonia N/100 ml. and the rate of flow be taken as 200 ml./min., the absorption of ammonia amounts to 4·3 g. N/day. For comparison, values for saliva may be taken as 5 l./day with 10 mg. urea N/100 ml. representing a salivary secretion of 0·5 g. N/day. Further, it appears that nitrogen, derived from the food, may in part be lost to the animal by this means and will thus constitute a factor in determining the biological value of the food protein. It is evident, too, that when urea is fed to the animal as a source of nitrogen, part at least will be absorbed from the fore stomachs as ammonia and hence be valueless to the animal.

SUMMARY

1. Ammonia occurs only in traces (if at all) in the blood of the general circulation of the sheep while the venous blood draining the rumen contains about 1·5 mg. ammonia N/100 ml.; it is, therefore, concluded that ammonia is absorbed from the rumen. Addition of a solution of ammonium acetate to the rumen after removal of the ingesta produces a prompt rise in the level of ammonia in the blood draining the organ. It is calculated that the quantity of ammonia N absorbed from the rumen may be of the order of 4–5 g./day.

2. Urea occurs in significant quantities in the saliva of the sheep. Attention is drawn to the circulation of nitrogen in the normal digestive process, viz. urea in saliva is converted in the rumen into ammonia, which may be absorbed into the portal blood stream and converted by the liver to urea, which again becomes available for secretion in the saliva.

3. Ammonia derived from the deamination of proteins in the feed contributes an important fraction of the nitrogen of the ruminal ingesta.

I wish to express my thanks to the Agricultural Research Council for a grant covering the cost of this research.

REFERENCES

Barcroft, J., McAnally, R. A. & Phillipson, A. T. (1944). *J. exp. Biol.* **20**, 120.

Conway, E. J. (1947). *Microdiffusion Analysis and Volumetric Error*, 2nd ed. London: Crosby, Lockwood and Son Ltd.

Conway, E. J. & Cooke, R. (1939). *Biochem. J.* **33**, 457.

Conway, E. J. & O'Malley, E. (1942). *Biochem. J.* **36**, 655.

Folin, O. & Denis, W. (1912). *J. biol. Chem.* **11**, 161.

Harris, L. E., Work, S. H. & Henke, L. A. (1943). *J. Anim. Sci.* **2**, 328.

Hench, P. S. & Aldrich, M. (1922). *J. Amer. med. Ass.* **79**, 1409.

Hench, P. S. & Aldrich, M. (1923). *J. Amer. med. Ass.* **81**, 1997.

Komarov, S. A. & Stavraky, G. W. (1940). *Canad. J. Res.* **18**, 233.

Koprowski, H. & Uninski, H. (1939). *Biochem. J.* **33**, 747.

Lenkeit, W. & Becker, M. (1938). *Z. Tierernähr.* **1**, 97.

McClymont, G. L. (1946). *Aust. vet. J.* **22**, 95.

Mangold, E. & Schmitt-Kramer, C. (1927). *Biochem. Z.* **191**, 411.

Parnas, J. K. & Klisiecki, A. (1926). *Biochem. Z.* **173**, 224.

Scheunert, A. & Krzywanek, Fr. W. (1930). *Pflüg. Arch. ges. Physiol.* **223**, 472.

Scheunert, A. & Trautmann, A. (1921). *Pflüg. Arch. ges. Physiol.* **192**, 33.

Schmitz, H. W. (1923). *J. Lab. clin. Med.* **8**, 78.

Schwarz, C. & Steinlechner, H. (1925). *Biochem. Z.* **156**, 130.

Smith, J. A. B. (1945). *Proc. Nutr. Soc.* **3**, 203.

Stephenson, M. (1939). *Bacterial Metabolism*, 2nd ed. London: Longmans, Green and Co.

Wegner, M. I., Booth, A. N., Bohstedt, G. & Hart, E. B. (1941). *J. Dairy Sci.* **24**, 51.

The Biochemistry of Bacterial Toxins

2. THE ENZYMIC SPECIFICITY OF *CLOSTRIDIUM WELCHII* LECITHINASE

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The general properties of a lecithinase present in the toxic culture filtrates of *Clostridium welchii* were described by Macfarlane & Knight (1941), who concluded that this enzyme was probably identical with *Cl. welchii* alpha toxin, the main lethal component of these filtrates. Since none of the *Cl. welchii* toxins examined hydrolyzed diphenyl-, monophenyl- or β-glycero-phosphate, or nucleic acid, this enzyme appeared to be a true lecithinase, as distinct from a phosphodiesterase, although the linkage attacked is of the diester type leading to the formation of phosphorylcholine and a diglyceride. In a further study of the enzyme specificity, Macfarlane (1942) reported briefly that kephalin was not decomposed by *Cl. welchii* toxin, but sphingomyelin was slowly hydrolyzed; the products of this hydrolysis, however, were not isolated.

Recently Zamecnik, Brewster & Lipmann (1947), who were unaware of this finding, examined the specificity of the toxin by an elegant adaptation of the manometric technique, and stated that it was inactive towards sphingomyelin, as well as to the other phospholipins tested. It seemed possible that the negative result of these authors with sphingomyelin was due to the short reaction time and low toxin concentration employed, but it was desirable to confirm the earlier positive results in more detail, since the specificity of the lipolytic activity of the toxin is a matter of some interest. *Cl. welchii* toxins in general appear to be free from phosphoesterases, lipase and nuclease, and the specificity of action might therefore be exploited for the differential estimation of phospholipins, for the elucidation of phospholipin structure or for the stepwise degradation of lipoprotein compounds.

The sample of sphingomyelin used in these experiments was kindly given to me by Dr O. Rosenheim, as one which, from its method of preparation as an ether-insoluble fraction, was free from lecithin; it contained, however, about 25 % cerebroside. Several hydrolyses of this crude sphingomyelin with *Cl. welchii* toxin have been carried out, in which from 70 to 90 % of the phosphorus present was converted into a water-soluble compound, with a satisfactory recovery of phosphorylcholine. It proved difficult, however, owing to the overlapping solubilities in the solvents tried, to separate the fatty product, which on the classical formula of sphingomyelin should be lignocerylsphingosine, from the accompanying cerebroside in sufficient yield and purity to characterize the product, though evidence of the formation of a nitrogenous product free from phosphorus and carbohydrate was obtained. It is clear also, since the starting material may be a mixture of 'sphingomyelins', that the hydrolysis product may be a mixture containing a variety of fatty acid residues,

quite apart from any cerebroside impurity in the crude lipin.

Dr Lipmann has since informed me that his early data (manometric) showed a very slow reaction between the toxin and sphingomyelin, which was attributed to contamination of the sphingomyelin with a little lecithin, but that using larger amounts of toxin and a longer reaction time he has confirmed the decomposition of sphingomyelin. It appears therefore that *Cl. welchii* toxins have a curious specificity, which is presumably due to a single enzyme, in splitting off phosphorylcholine from lecithin and sphingomyelin, though neither kephalin (phosphatidylethanolamine or phosphatidylserine) nor, according to Zamecnik *et al.* (1947), glycerophosphorylcholine nor lysolecithin are attacked.

EXPERIMENTAL

Methods. The methods used for testing the enzymic activity of the toxin were similar to those previously described (Macfarlane & Knight, 1941), the reactions being followed by estimation of the acid-soluble organic P formed. One enzyme (lecithinase) unit (e.u.) = the amount producing 0·1 mg. P from excess lecithin at pH 7·6 and 37° in 15 min. under standard conditions.

Toxin. Glycerinated or dried toxin derived from *Cl. welchii*, type A, strain S 107, was used for the study of reaction rates, and a highly purified dry preparation, 354 H, for which I am indebted to Dr van Heyningen and Miss Bidwell, for the isolation experiments.

Substrates. The crude sphingomyelin used contained 2·7 % P, 2·5 % N, and 5·0 % galactose by orcinol estimation; calculated from this, the sample contained approximately 71 % of sphingomyelin and 23 % of cerebroside.

'Lecithin' was prepared from egg yolk, by repeated precipitations of the portion soluble in ether at 0° with acetone. The product usually contained 80–90 % lecithin.

A mixed preparation of 'lecithin-kephalin' was obtained from acetone-dried sheep brain by extraction with ether and repeated precipitation with acetone of the ether-soluble portion.

A purer preparation of 'kephalin' was obtained by repeated precipitation of the ethereal extract with 4 vol. ethanol.

Hydrolysis of sphingomyelin

Rate of hydrolysis. A representative protocol showing the comparative rate of hydrolysis of sphingomyelin and lecithin is shown in Table 1. At the same concentration, the hydrolysis of sphingomyelin is considerably slower than that of lecithin, but proceeded to the extent of 70 % in the conditions used. The hydrolysis, like that of lecithin, is activated by Ca ions and inhibited by NaF.

Isolation of products: Procedure (a). Crude lipin (0·69 g.) was emulsified in 25 ml. 0·02 M-$CaCl_2$, the pH adjusted to 8·4 with NaOH, and 10 mg. (600 e.u.) *Cl. welchii* toxin 354 H added in three portions at intervals of 3 hr. The mixture was incubated at 37°, being titrated back to pH 7·4 at intervals with 0·05 N-NaOH and finally to pH 8·4. The hydrolysis was allowed to proceed for 22 hr. in all, at which point 86 % of the P was water-soluble.

The aqueous portion was separated by filtration, evaporated to a small bulk on a water bath, refiltered, evaporated to dryness and re-extracted into 2 ml. water from which the crystalline Ca compound of phosphorylcholine was obtained on addition of ethanol. (Found: P, 9·1; N, 3·7; Cl, 10·1 %. Calc. for $C_5H_{13}O_4NPClCa.4H_2O$: P, 9·46; N, 4·25; Cl, 10·75 %.) The identity was further confirmed by determination of the hydrolysis constant in N-HCl at 100°, and by the rapid and complete hydrolysis of this phosphoric ester by bone phosphatase (phosphomonoesterase) to orthophosphate with liberation of choline.

The water-insoluble portion was extracted with cold acetone, dried and re-extracted with acetone. The total acetone-soluble material amounted only to 40 mg.; assuming that this material was a diglyceride derived from lecithin, the maximum amount of lecithin possibly present as an impurity in the crude sphingomyelin corresponded to less than 10 % of the organic P present. The acetone-insoluble material was fractionated by deposition from ethanolic solutions at different temperatures, the course of fractionation being followed by the P, N and carbohydrate content, as shown in Table 2.

Isolation of products: Procedure (b). Another hydrolysis, using 0·5 g. crude sphingomyelin, was carried out in a similar way, but the water- and acetone-insoluble material was extracted with cold chloroform. The fractions soluble and insoluble in chloroform, redeposited from ethanolic

Table 1. *Comparative hydrolysis of lecithin and sphingomyelin at* 37°

(Reaction mixtures contained 1·0 ml. aqueous emulsion of phospholipin (100 μg. P), 1 ml. borate buffer (Palitzsch), pH 7·6, and the additions below in a total volume of 6·0 ml.)

Cl. welchii toxin (Enzyme units)	Phospholipin	Additions	Time of incubation (min.)	P hydrolyzed (μg.)
1·1	Sphingomyelin	Nil	30	15
1·1	Sphingomyelin	$CaCl_2$	30	20
1·1	Sphingomyelin	$CaCl_2$	180	37
1·1	Sphingomyelin	NaF	180	12
1·1	Lecithin	Nil	30	30
1·1	Lecithin	$CaCl_2$	30	51
1·1	Lecithin	$CaCl_2$	180	76
1·1	Lecithin	NaF	180	21
3·3	Sphingomyelin	$CaCl_2$	180	71
3·3	Sphingomyelin	NaF	180	19

Table 2. *Balance sheet of the hydrolysis of crude sphingomyelin by* Cl. welchii *toxin*

	Quantities (mg.)			Composition (%)				
	Wt.	P	N	P	N	Carbohydrate (as galactose)	C	H
Original material	579	15·6	14·5	2·7	2·5	5·0	—	—
Calculated sphingomyelin (from P)	410	15·6	14·1	3·79	3·42	Nil	—	—
Calculated phrenosin (from galactose)	133	—	2·2	Nil	1·70	21·7	69·6	11·2
Calculated yield lignoceryl sphingosine	319	—	7·0	Nil	2·21	Nil	77·4	12·7
Found								
(1) P equivalent of acid formed	—	12·8	—	—	—	—	—	—
(2) Water-soluble portion	—	13·5	6·5	—	—	—	—	—
Cryst. phosphoryl choline	110	10·6	4·1	—	—	—	—	—
(3) Water-insoluble portion:								
Fraction from ethanol at 20°	86	1·65	1·46	1·92	1·7	9·2	—	—
Fraction from 95% ethanol at 20°	138	0	2·88	0	2·1	1·7	—	—
Fraction from ethanol at 0°	129	0·21	2·45	0·16	1·9	12·0	—	—
Mother liquors	83	0	—	0	—	4·0	—	—
Semi-crystalline material from pooled carbohydrate-low fractions from four hydrolyses	250	—	—	0	1·9	3·0	74·9	11·9

Table 3. *Hydrolysis of monoaminomonophosphatides by* Cl. welchii *toxin*

(Reaction mixture (6·0 ml.): 0·48 mg. lipin P; 3·0 e.u. toxin; 0·002 M-$CaCl_2$; pH 7·6, at 37°.)

Phospholipin	Amino N / Total N (%)	α-Amino N / Total N (%)	Calc. % of P as lecithin	% hydrolysis of P			
				1 hr.	2 hr.	3 hr.	4 hr.
(1) Ether-soluble acetone-insoluble (egg yolk)	22	Nil	78	70	74	78	78
(2) Ether-soluble acetone-insoluble (sheep brain)	83	23	17	17	19	22	22
(3) Ether-soluble ethanol-insoluble (sheep brain)	95	25	5	0	<5	<5	—

solution at 0°, contained 5·5 and 14·5% carbohydrate respectively, but repetition of this step on the carbohydrate-low fraction did not effect a further separation. This material was then fractionated by deposition from hot ethanol; the main fraction, which separated between 22° and 0°, contained P, 0·07; N, 1·9; carbohydrate, 4·5%.

Four hydrolyses of 0·5–0·6 g. crude sphingomyelin were carried out in all, in which from 67 to 91% of the lipin P was converted to a water-soluble form, with a satisfactory recovery of phosphorylcholine, while approx. 30–40% of the calculated sphingosine N of the phospholipin was recovered in a P-free carbohydrate-low fraction containing 1·9–2·1% N. This pooled material was insoluble in cold ethanol, acetone or ether, dissolving readily on warming in ethanol or acetone and redepositing on cooling. After several fractionations, the deposit from 95% aqueous ethanol was a semi-crystalline mass of irregular plates but still gave a reaction on heating with orcinol and H_2SO_4 which indicated the presence of 2–3% carbohydrate (Table 2). In view of the possibility that the starting material contained a mixture of 'sphingomyelins', as well as the cerebroside impurity, the further identification by degradation of this small amount of material was not attempted.

Action of Cl. welchii *toxin on kephalin*

The action of *Cl. welchii* toxin on individual monoaminomonophosphatides was determined by comparison of the maximal production of water-soluble P from ether-soluble phospholipins prepared from egg yolk and brain with the amino N and α-amino N of the sample. Table 3 shows that the maximum percentage hydrolysis of P corresponded with the percentage content of non-amino (lecithin) N, while practically no hydrolysis was observed in a 'kephalin' fraction from sheep brain containing 25% of the total N as α-amino N. The conclusion drawn from this result, that neither phosphatidylethanolamine nor phosphatidylserine was hydrolyzed by *Cl. welchii* lecithinase, has been confirmed by Zamecnik *et al.* (1947) using pure samples of these phospholipins.

SUMMARY

1. The action of *Cl. welchii* toxin on a sample of crude sphingomyelin has been examined. Approximately 90% of the lipin P was converted into a water-soluble form; the hydrolysis is slower than that of lecithin P in similar conditions, but like that of lecithin is activated by Ca ions and inhibited by NaF. Phosphorylcholine was recovered quantitatively from the hydrolysis products, together with a P-free product approximating to the composition of lignocerylsphingosine, which was not obtained pure.

2. The maximum hydrolysis of lipin P in mixed lecithin-kephalin preparations corresponded with the percentage content of non-amino (lecithin) N.

3. It is concluded, from the manner of hydrolysis, that *Cl. welchii* lecithinase hydrolyzes sphingomyelin, as well as lecithin, with production of phosphorylcholine, but does not attack phosphatidylethanolamine nor phosphatidylserine.

REFERENCES

Macfarlane, M. G. (1942). *Biochem. J.* **36**, iii.
Macfarlane, M. G. & Knight, B. C. J. G. (1941). *Biochem. J.* **35**, 884.
Zamecnik, P. C., Brewster, L. E. & Lipmann, F. (1947). *J. exp. Med.* **85**, 381.

The Biochemistry of Bacterial Toxins

3. THE IDENTIFICATION AND IMMUNOLOGICAL RELATIONS OF LECITHINASES PRESENT IN *CLOSTRIDIUM OEDEMATIENS* AND *CLOSTRIDIUM SORDELLII* TOXINS

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Nagler (1939) observed that when *Clostridium welchii* type A was grown in human serum an opalescence developed and eventually a layer of fat rose to the surface. Macfarlane, Oakley & Anderson (1941) found that *Cl. welchii* toxin acts on a clear emulsion of egg yolk to give a similar but more marked opalescence, and that this reaction is due entirely to the α-toxin, the main lethal component of *Cl. welchii* type A culture filtrates; this is termed the lecithovitellin (L.V.) test. Subsequently, *Cl. welchii* toxin was found to contain a lecithinase (Macfarlane & Knight, 1941) which is probably identical with the agent responsible for the Nagler and L.V. reaction, and therefore with the α-toxin.

Hayward (1941, 1943) and Crook (1942) found that other species of *Clostridium*, particularly *Cl. oedematiens*, and also some aerobic spore bearers, may give positive Nagler or L.V. tests; this fact, as pointed out by Hayward (1943), does not militate against the specificity of the test for the detection of *Cl. welchii*, as the essence of the test is not merely a positive reaction but the inhibition of such a reaction by the specific antitoxin. There is, however, a tendency to regard any bacterial agent giving a positive L.V. test as a 'lecithinase', which is clearly unwarrantable without chemical evidence and may lead to confusion on the nature of the bacterial agents.

Macfarlane (1942) briefly reported the identification of a lecithinase in *Cl. oedematiens* toxins, which was immunologically distinct from the main lethal component, and also from *Cl. welchii* lecithinase. Oakley, Warrack & Clarke (1947), in a comprehensive study of the antigenic components of *Cl. oedematiens* toxins, found that the L.V. factor (γ-toxin) in *Cl. oedematiens* type A filtrates was immunologically distinct from that (β-toxin) present in type B filtrates. Through the courtesy of Dr Oakley, I have been able to show that the immunological behaviour of the lecithinases present in each of these types was similar to that of the L.V. factor, thus indicating that *Cl. oedematiens* β- and γ-toxins are lecithinases. The identification of these lecithinases, and of a lecithinase present in culture filtrates of certain strains of *Cl. sordellii* or *Cl. bifermentans*, is described below.

EXPERIMENTAL

The general methods of procedure and analysis were similar to those used by Macfarlane & Knight (1941). Lecithinase activity was determined by estimation of acid-soluble P liberated from lecithin, and the L.V. factor by the development of turbidity in a clear emulsion of egg yolk.

Toxins and antitoxins

Cl. oedematiens. For the initial experiments in 1942, dry or glycerinated toxins derived from *Cl. oedematiens* 'Albiston' were used. This type B strain toxin was in routine use in the Serum Department of the Lister Institute at Elstree for the hyperimmunization of horses; the various antitoxic sera from Elstree examined for antilecithinase activity were B type (anti-β-toxin). *Cl. oedematiens* type B toxin, O.C. 91044, with corresponding antisera Todd 3 and Todd 61, and type A toxins, A.E. 327, and O.C. 19345 with antisera 202 and 645 were given by Dr Oakley.

Cl. sordellii (or *bifermentans*). Culture filtrates were prepared in this laboratory from five strains (referred to for convenience as *Cl. sordellii*) obtained from Miss Hayward, described as: A 127 A (*sordellii* or *bifermentans*) from necrotic wound; N.C.T.C. 2919, *sordellii*; A 90 *b*, *sordellii*, from a case of gas gangrene; A 112 (*sordellii* or *bifermentans*); 7851 *a* (*sordellii* or *bifermentans*) from a gangrenous wound.

The strains were subcultured on Robertson's meat medium, and inoculated into the peptone medium described by Macfarlane & Knight (1941). After growth at 37° for 4–18 hr., the fluid was cleared by centrifuging and tested immediately for enzyme activity. The lethality in mice was not tested. *Cl. sordellii* antitoxin was not available.

Expression of results

One lecithinase or enzyme unit (e.u.) is that amount producing 0·1 mg. acid-soluble P from lecithin in 15 min. in standard conditions (Macfarlane & Knight, 1941). One egg yolk unit (y.u.) is that amount producing the same opacity under standard conditions as a test dose of *Cl. welchii* toxin containing one e.u. The unit of *Cl. oedematiens* α-antitoxin (anti-lethal) is the International Unit, those for β- and γ-antitoxin are those defined by Oakley *et al.* (1947) with reference to selected sera.

The antilecithinase activity of *Cl. oedematiens* antitoxin sera is calculated on the basis that 0·04 unit causes 50 % reduction in the hydrolysis under standard conditions by a test dose of *Cl. oedematiens* toxin containing 2 e.u. lecithinase. This was adopted because 0·04 unit of *Cl. welchii* antilecithinase (the Elstree Standard no. 1 *Cl. welchii* antitoxin having been arbitrarily selected by Macfarlane & Knight as a standard containing 95 units/ml.) causes approximately 50 % reduction in the hydrolysis by 2 e.u. of *Cl. welchii* lecithinase.

These *Cl. welchii* and *Cl. oedematiens* antilecithinase units are therefore comparable in a biochemical sense; but since the inhibiting power of the antilecithinase will depend on the total amount of antigen (toxin or toxoid) present in a particular sample of toxin, the *Cl. oedematiens* antilecithinase unit is not a fixed standard against all toxins. As the *Cl. oedematiens* type A and type B lecithinases are probably identical with the γ- and β-toxins respectively, the antilecithinase activity should preferably be expressed in future in γ- and β-units by comparison of the inhibiting power with that of the standard sera against the same test dose of toxin.

Hydrolysis of lecithin by Cl. oedematiens *toxin*

Though the liberation of acid-soluble P from lecithin by *Cl. oedematiens* toxin can readily be demonstrated, the quantitative recovery of the hydrolyzed products was more difficult than in *Cl. welchii* hydrolysis, for the low lecithinase activity of the toxins involved either the addition of a large amount of impurities or the prolongation of the hydrolysis, with the risks of autolysis or secondary reactions by associated enzymes.

Identification of products of hydrolysis by type B toxin. The following protocol is representative of a number of hydrolyses.

Egg lecithin (0·5 g. containing 18 mg. P) was emulsified in 15 ml. 0·01 M-$CaCl_2$, the pH adjusted to 7·4 and 15 mg. *Cl. oedematiens* type B toxin (4·5 e.u.) added. The hydrolysis was carried out at 37°, more enzyme being added at 24 hr. and the pH adjusted to 7·4 with NaOH at intervals. After 45 hr., 0·56 mmol. acid had been liberated, equivalent to 95 % hydrolysis. The mixture was then separated into water-soluble and ether-soluble fractions by shaking several times with ether.

The aqueous solution, which contained 91 % of the original lipin P as organic P, was evaporated to dryness and the crystalline Ca component of phosphorylcholine obtained from the ethanolic extract of the residue. (Found: P, 9·32; N, 3·7; Cl, 11·4; H_2O, 21·6 %. Calc. for $C_5H_{13}O_4NClPCa.4H_2O$: P, 9·46; N, 4·25; Cl, 10·75; H_2O, 21·86 %.) The yield in the first crop was low, only 25 % of that calculated from the organic P liberated; in subsequent hydrolyses the yield of crystalline material was better, up to 60 %.

The ethereal solution was evaporated and the residue was extracted with acetone. The dry weight (0·310 g.) of acetone-soluble material was equivalent to an 85 % recovery of a diglyceride, calculated as palmityloleylglyceride; it had an acid value 2·7 and saponification value 176 (calculated 179).

Hydrolysis by type A toxin. (1) A lecithin emulsion containing 21·0 mg. lipin P in 0·02 M-$CaCl_2$ was treated with 20 mg. type A toxin O.C. 19345 at pH 7·4 and 37°, a further 20 mg. enzyme being added after 4 and 20 hr. The hydrolysis was stopped after the liberation of one equivalent of acid and the fatty portion separated from the aqueous solution by extraction with ether. The ethereal solution was evaporated and the residue extracted with acetone, leaving some acetone-insoluble material. The acetone-soluble material (380 mg.), dissolved in ethanol, had a saponification value of 167 but an acid value of 20.

The aqueous solution contained only 15·9 mg. P (76 % of the calculated amount) of which 14·1 mg., all as organic P, were recovered on treatment of the evaporated and filtered solution with $CaCl_2$ and ethanol. All the organic P in a test sample was rapidly hydrolyzed by bone phosphatase, indicating the presence of a monophosphoric ester. The material was redeposited, on addition of ethanol to the aqueous solution, in a more or less crystalline state similar to that of the crystalline Ca compound of phosphorylcholine, but the analysis indicated some impurity. (Found: N, 3·4; Cl, 7·2; P, 8·1 %. Calc. for $C_5H_{13}O_4NClPCa.4H_2O$: N, 4·25; Cl, 10·75; P, 9·46 %.)

(2) The results suggested that the type A toxin contained both a lecithinase splitting off phosphorylcholine, and a lipase attacking either lecithin or the diglyceride formed by the primary action of the lecithinase. A second hydrolysis was carried out on an emulsion containing 17 mg. lipin P, in similar conditions but with 50 mg. toxin initially. The hydrolysis was stopped after 47 hr., when 2·3 equivalents of acid had been liberated. The mixture was acidified with HCl and extracted with ether, the ethereal and aqueous solutions being washed with water and ether respectively.

The aqueous solution contained 13 mg. P (76 % of the original lipin P), all in an organic form which was rapidly hydrolyzed by bone phosphatase. A semi-crystalline Ca compound was obtained on treatment of the concentrated solution with ethanol, which had a P : N ratio 1 : 0·9; the analysis again indicated the presence of some impurity.

The ethereal solution on evaporation gave 314 mg. fatty material, which in ethanolic solution required 4·95 ml. 0·1 N-alkali for neutralization, equivalent to an acid value of 88·5 or approximately 140 mg. free fatty acid as oleic or palmitic acid. The neutral fat and the soap were separated by extraction with ether, and the content of saturated and unsaturated acids in each determined approximately by one separation of the Pb salts in 95 % ethanol.

The fatty acids (138 mg.) recovered from the soap gave 85 mg. insoluble Pb salt, equivalent to 60·6 mg. palmitic acid, while 70 mg. fatty acid were recovered from the soluble Pb salt.

The neutral fat (161 mg.) gave 144 mg. acetone-soluble fat, with an acid value less than 4, which was saponified in ethanolic KOH. The saponification value of 159 was approximately that of oleyl or palmityl monoglyceride (156–157); 106 mg. fatty acid were recovered yielding 56 mg. insoluble Pb salts (equivalent to 40 mg. palmitic acid), and 65 mg. fatty acid from the soluble Pb salts.

In both these hydrolyses, therefore, more than 70 % of the lipin P was recovered as a monophosphoric ester with a P : N ratio of approximately unity, and there seems little doubt that *Cl. oedematiens* type A toxin contains a lecithinase similar in action to that of type B toxin and *Cl. welchii* toxin, which decomposes lecithin into phosphorylcholine and a diglyceride. Type A toxin contains in addition a lipase which can split off both saturated and unsaturated fatty acids, either from the diglyceride or possibly from lecithin itself. In the latter case the products of hydrolysis by this second enzyme alone might include a monofatty-acid derivative of glycerophosphorylcholine, as well as glycerophosphorylcholine. The ratio of all the products formed by the toxin would naturally be dependent on the relative activities of the two enzymes, but the difficulty experienced in isolating analytically pure phosphorylcholine suggests that, though the γ-toxin is predominant, traces of other primary products of lecithin were formed.

The identification of two lipolytic enzymes in *Cl. oedematiens* type A toxin—a lecithinase (γ-toxin) similar in action to *Cl. oedematiens* β-toxin and to *Cl. welchii* α-toxin, and a lipase splitting off fatty acids—is consistent with the morphological appearance of type A strains in plate tests and the identification of various components in the culture filtrates by immunological tests. Nagler (1945) showed that the surface of colonies of *Cl. oedematiens* type A, grown on egg-yolk blood agar, is covered with a 'pearly layer', while type B strains do not produce this effect. Oakley *et al.* (1947) distinguished in type A culture filtrates two components producing opalescence in egg yolk: the γ-toxin and a second factor probably responsible for the 'pearly layer', named ϵ-toxin. It seems reasonable to suppose that the action of the ϵ-toxin is that of the lipase forming a pearly layer of fatty acid upon the colony, but further work is required to identify this enzyme by the inhibitory action of anti-γ and anti-ϵ sera, and to characterize its biochemical nature.

Effect of pH, *activators, etc. Cl. oedematiens* lecithinase (β-toxin) is active over a wide pH range with a broad optimum from 6·0 to 7·6. The pH activity curve was not determined with great accuracy, as it differed somewhat in shape with different toxins, which were all crude.

The enzymic activity was in general increased by the addition of $CaCl_2$ to the reaction mixture and inhibited by addition of NaF. These crude toxins were, however, active in the absence of added Ca, probably owing to the presence of sufficient Ca in the toxin and lecithin.

Lecithinase content of various toxins. The enzymic activity/unit weight or unit volume was determined by estimation of acid-soluble organic P liberated by measured amounts of the toxin in the following conditions: 1 ml. borate buffer (Palitzsch), pH 7·1; 0·2 ml. 0·3 M-$CaCl_2$; 1 ml. 2·5 % aqueous lecithin; toxin or water to 6·0 ml.; incubated at 37° for 15 min.; acidified with 1·0 ml. 10 % trichloroacetic acid and filtered.

Table 1 shows the lecithinase activity of various toxic culture filtrates, or dry toxins prepared from these, together with the lethal capacity (LD_{50}) in mice. The lecithinase activity of *Cl. oedematiens* toxins was very much lower than that of *Cl. welchii* toxins, and varied independently of the main lethal component; *Cl. oedematiens* type A toxins appeared generally to be lower in activity than type B toxins.

Table 1. *Lecithinase activity* (*e.u.*) *and lethality* (LD_{50}) *of* Cl. welchii *and* Cl. oedematiens *toxins*

Toxin	Values/mg. dry or ml. fluid toxin: e.u.	LD_{50}	Ratio LD_{50}/e.u.
Dry toxins			
Cl. welchii, type A, W.E. 5	12·2	25	2
Cl. oedematiens, type A, A.E. 327	0·06	—	—
Cl. oedematiens, type A, A.E. 208	0·10	—	—
Cl. oedematiens, type A, O.C. 19345	0·15	—	—
Cl. oedematiens, type B, Oe I	0·12	200	1,670
Cl. oedematiens, type B, Oe II	0·30	—	—
Cl. oedematiens, type B, 12XB	0·29	—	—
Fluid toxins			
Cl. welchii, type A, K. 178	210	400	1·9
Cl. welchii, type A, K. 188	294	450	1·5
Cl. oedematiens, type B, V.F.I	1·5	6,000	3,900
Cl. oedematiens, type B, K.I	1·0	12,000	12,250
Cl. oedematiens, type B, O.C. 91044	1·4	—	—

Antilecithinase activity of Cl. oedematiens *antitoxic sera*

The general method for measuring the inhibiting action of antitoxic sera was as follows: 1·0 ml. of toxin containing 2 e.u. was allowed to combine with two or three different amounts of antitoxic sera in a total volume of 3·8 ml. for 10 min. at room temperature; borate buffer, $CaCl_2$ and lecithin were added and the hydrolysis measured in the usual way. The percentage inhibition of the hydrolysis by the same test dose of toxin without antitoxin in the same test was plotted against the amount of antitoxin, the amount equivalent to 50% inhibition being regarded as containing 0·04 antilecithinase units. Using homologous antitoxins which produced complete inhibition in excess, an approximately linear relationship obtained in the above conditions.

ratio of its β- and γ-antitoxin content. It seems fair to conclude that the type A and type B lecithinases are identical respectively with the γ- and β-toxins distinguished by Oakley *et al.* (1947).

A protocol (Table 3) shows that, while there was practically no inhibition of the β-lecithinase by the type A serum, there was complete inhibition of the γ-lecithinase by the type B serum (Todd 3) if sufficient serum was added. Inspection of the titres for various sera given by Oakley *et al.* (1947, Table 8) shows that the ratio γ/β-units in a β-rich serum was always considerably higher than the ratio β/γ-units in a γ-rich serum. This is probably not a genuine cross reaction between β-antitoxin and γ-lecithinase, but is due simply to the fact that all the horses had received a preparatory dose of alum-precipitated toxoid from a type A (γ-toxin) filtrate; it is at least unlikely that complete inhibition in the lecithinase test, which in contrast to the egg-yolk

Table 2. *Antilecithinase titres of* Cl. oedematiens *type A and type B antisera against type B and type A lecithinase*

(1 e.u. of toxin A.E. 327 = 17 mg. ≡ 8 test doses for L.V. test. 1 e.u. of toxin O.C. 91044 = 0·7 ml. ≡ 31 test doses for L.V. test.)

Serum	Antilecithinase units/ml. serum against 1 e.u. test toxin: Type A toxin A.E. 327	Type B toxin O.C. 91044	Antitoxin titres (L.V. units/ml.): γ-units	β-units
Type A 202	5·0	<0·2	700	0·5
644	7·7	—	700	2·0
Type B Todd 3	0·4	40	120	2500
Todd 61	0·2	—	65	450

A number of type B antitoxic horse sera prepared by routine immunization, and containing from 750 to 2500 international units/ml., were tested against the type B toxin Oe II and found to contain small amounts of antilecithinase (0·2–3·0 units/ml.). The ratio i.u./antilecithinase units varied from 250 to 10,000; thus the antilecithinase is clearly distinct from the anti-lethal toxin (Oakley's α-antitoxin).

Immunological specificity of type A and type B antilecithinase. The cross reactions between type A and type B antitoxic sera and type B and type A lecithinases were tested in a similar manner, but with a lower test dose of toxin (1 e.u.) owing to the small amounts available. The approximate antilecithinase titres against the same number of e.u.'s of the homologous and heterologous lecithinases are shown in Table 2, together with the titres for β- and γ-antitoxin determined by Dr Oakley. There is clearly a marked difference between the inhibiting power against the homologous and the heterologous enzyme. Considering the limits of accuracy of the tests, and the different basis of testing, there is also a substantial agreement between the ratio of the antilecithinase titre of a particular serum against the homologous and the heterologous toxin, and the test is augmented rather than inhibited by non-specific protein, could be due to non-specific inhibition by a low-titre serum.

Table 3. *Inhibition of type A lecithinase by type B antisera*

Toxin	Antiserum (ml.)	Hydrolysis (% of test dose)
Type A		
Test dose 1 e.u. γ-lecithinase	Type A 0·004	68
	0·01	32
	0·02	10
	Type B 0·007	96
	0·033	79
	0·10	49
	0·20	16
Type B		
Test dose 1 e.u. β-lecithinase	Type A 0·004	94
	0·02	93
	Type B 0·001	55
	0·007	0
	0·02	0

Species specificity of Cl. welchii *and* Cl. oedematiens *lecithinase.* The cross reactions of *Cl. welchii* type A and *Cl. oedematiens* type B antitoxins with the

heterologous enzymes were examined in a similar way. There was practically no inhibition of either of the enzymes by the heterologous antitoxin, in amounts greatly exceeding those inhibiting the homologous enzyme.

Lecithinase activity of Cl. sordellii *culture filtrates*

Egg-yolk and lecithinase tests. Filtrates of 23 hr. cultures of five strains of *Cl. sordellii* were tested for their capacity to produce turbidity in a clear emulsion of egg yolk, in comparison with a test dose (1 e.u.) of *Cl. welchii* toxin. Three of the strains gave a positive reaction (N.C.T.C. 2919, A 90 B, A 112) with values 1·0, 0·9 and 0·5 y.u./ml. The first two filtrates were tested for lecithinase by estimation of acid-soluble P liberated from lecithin, and gave values of 0·7 e.u./ml. In various tests on culture filtrates from these strains, the lecithinase activity was lower, to a variable extent, than the egg-yolk value, indicating the presence of a second factor affecting egg yolk. As the routine test for lecithinase was complicated by the presence of inorganic P in a culture filtrate of low activity, an attempt was made with one filtrate to purify the enzyme by salting it out, but the dialyzed solution showed an even greater discrepancy between the egg factor (3 y.u./ml.) and the lecithinase (0·9 e.u./ml.).

Decomposition of egg yolk. The decomposition of egg yolk by a *Cl. sordellii* filtrate and by *Cl. welchii* toxin was followed with reference both to the conversion of lipin P to an acid-soluble form, and to the liberation of acid-soluble N. Table 4 shows that almost all the egg-yolk P, including presumably the bound lipin P as well as the ether-soluble P, was converted into acid-soluble organic P; there was slightly more formation of acid-soluble N by *Cl. sordellii* than by *Cl. welchii* toxin. It is possible that *Cl. sordellii* cultures contain an enzyme attacking the yolk protein and thereby affecting the turbidity development in the egg-yolk test, but it seems more probable that this toxin, like the *Cl. oedematiens* type A toxin, contains a lipase, for *Cl. sordellii* also gives a 'pearly layer' in plate tests. The products of hydrolysis of lecithin by *Cl. sordellii* toxin were not identified, but the action of the lecithinase is apparently similar to that of *Cl. welchii* in so far as an acid-soluble phosphoric ester is produced.

Inhibition by heterologous antitoxic sera. Owing to their low lecithinase activity, the culture filtrates from these strains of *Cl. sordellii* were not suitable for tests of the antilecithinase activity of sera, but some tests were carried out with the egg-yolk test. With one filtrate, inhibition either by *Cl. welchii* or *Cl. oedematiens* antitoxic sera was maximal at 50%; with other filtrates complete inhibition was sometimes observed. Further work is required on purified and concentrated preparations of *Cl. sordellii* lecithinase to determine whether this inhibition is a true partial inhibition of the lecithinase, or a specific inhibition of a second factor affecting egg yolk, or a non-specific inhibition of the development of turbidity by the excess protein added. So far as it goes, the result is in accord with Hayward's (1943) finding in plate tests, that the Nagler reaction of some strains of *Cl. bifermentans* or *Cl. sordellii* is completely inhibited, and that of other strains partially inhibited, by *Cl. welchii* antitoxin.

DISCUSSION

It has been shown previously that the lecithinase present in *Cl. welchii* toxin is probably identical with *Cl. welchii* α-toxin, the main lethal, necrotic and haemolytic component of *Cl. welchii* type A toxins. Evidence has now been presented that culture filtrates of *Cl. oedematiens* contain a lecithinase similar in biochemical action to *Cl. welchii* lecithinase, but quite distinct from the main lethal component. The lecithinases present in culture filtrates from *Cl. oedematiens* type A and type B strains are immunologically distinct from each other, and there seems little doubt that they are identical respectively with the γ- and β-toxins described by Oakley *et al.* (1947). These authors point out that, as lecithinases, β- and γ-toxins might be expected to be haemolytic, necrotic and lethal, and that their own failure to demonstrate a lethal effect with certainty, after adsorption of the main lethal component with an appropriate antitoxic serum, was probably due to the small amount of lecithinase present. Assay of the lecithinase activity of various *Cl. oedematiens*

Table 4. *Liberation of* P *and* N *from egg yolk by* Cl. welchii *and* Cl. sordellii *toxins*

(Reaction mixture: 10 ml. yolk emulsion (1·1 mg. total P) and 2 ml. toxin (2 e.u.) or saline; 4 hr. at 37°.)

	Found after incubation (mg.)		
Determination	Control, without toxin	With *Cl. welchii* toxin	With *Cl. sordellii* toxin
P in ether-soluble fraction	0·56	Nil	Nil
Ortho P in trichloroacetic acid-soluble fraction	Nil	Nil	Nil
Total P in trichloroacetic acid-soluble fraction	Nil	0·96	0·97
N in trichloroacetic acid-soluble fraction	Nil	0·90	1·35

filtrates shows that this explanation is probably correct, since the maximum content found (1·5 e.u./ml.) is only equivalent (by comparison with *Cl. welchii* lecithinase) to approximately 3 LD_{50} doses/ml. for the mouse, compared with 6000–1200 LD_{50} doses/ml. of the main lethal component (*Cl. oedematiens* α-toxin).

The immunological and enzymic specificities of the lecithinases present in *Cl. welchii* and *Cl. oedematiens* toxins offer some entertaining facts for those interested in the relationship between chemical configuration and biological action. It may be supposed that the group responsible for lecithinase activity is closely similar in the three enzymes (*Cl. welchii* α-toxin, *Cl. oedematiens* β- and γ-toxins); the fine configuration of *Cl. welchii* lecithinase is such that it decomposes lecithin and, probably but less readily, sphingomyelin, but not kephalin (Macfarlane, 1942, 1948) while the failure to attack lysolecithin and glycerophosphorylcholine (Zamecnik, Brewster & Lipmann, 1947) suggests that the presence of two fatty acids is essential for orientation of the substrate molecule. Each of these enzymes is immunologically distinct; since the antilecithinase groups of the specific antibodies are presumably as similar to each other as the lecithinase groups of the antigens, it may be inferred that the immunological distinction between these antibodies is conferred by a 'species' or 'type' group in the antigen. But in other instances the immunological distinction is presumably conferred by the enzymic or 'toxic' group of the antigen, e.g. the distinction between the antilecithinase, antihyaluronidase, anticollagenase, etc. of *Cl. welchii* antitoxic sera. Thus these bacterial enzymes carry in effect two specific antigenic configurations, e.g. '*W* + lecithinase' or 'Oe A + lecithinase'; whether these configurations are discrete or whether they are interlocked to form a unique configuration, the structure is such that it can combine specifically both with the substrate of the enzyme, and with a type-specific anti-enzyme.

The immunological specificity of these 'toxic' enzymes is no doubt a reflexion of the stereochemical obtrusiveness of the antigenic structure, which is not without bearing on the virulence of the toxins. Discussing the behaviour of toxic enzymes, Macfarlane & Knight (1941) said, 'The toxin can primarily affect only those cells which contain the appropriate substrate for its enzymic activity, *and then only if this substrate is accessible to it*'. The very interesting fact has been pointed out by Oakley *et al.* (1947) that sheep red cells are readily attacked by the lecithinase *Cl. welchii* α-toxin, but are insensitive to the lecithinases *Cl. oedematiens* β- and γ-toxins; the latter haemolyze horse red cells, which are not attacked by *Cl. welchii* α-toxin. One possible explanation of this phenomenon is that stereochemically the specific blood-group substance of the host permits or hinders the approach of the type-specific group of the lecithinase, and thus permits or prevents the orientation of the enzyme towards the lecithin of the red-cell membrane. A similar kind of interference may well be the basis of the relative 'susceptibility' or 'resistance' of different host species to a particular toxin. It is clear that the activity of a bacterial enzyme towards a biological structure cannot be predicted merely on the basis that the structure contains the necessary substrate.

SUMMARY

1. *Cl. oedematiens* culture filtrates contain a lecithinase similar in biochemical properties to that present in *Cl. welchii* filtrates.

2. The lecithinases present in *Cl. oedematiens* type A and type B filtrates are immunologically distinct from each other, and from *Cl. welchii* lecithinase, in that their activity is inhibited by a homologous but not inhibited by a heterologous antitoxic serum. The type A and type B lecithinase are probably identical respectively with the γ- and β-toxins of *Cl. oedematiens* filtrates.

3. *Cl. oedematiens* type A toxins also contain a lipase, which is possibly identical with the factor (ε-toxin) responsible for the 'pearly layer' effect in colonies of this organism.

4. Culture filtrates of certain strains of *Cl. sordellii* also contain a lecithinase which is not yet characterized.

REFERENCES

Crook, E. M. (1942). *Brit. J. exp. Path.* **23**, 37.
Hayward, N. J. (1941). *Brit. med. J.* **1**, 811.
Hayward, N. J. (1943). *J. Path. Bact.* **55**, 285.
Macfarlane, M. G. (1942). *Biochem. J.* **36**, iii.
Macfarlane, M. G. (1948). *Biochem. J.* **42**, 587.
Macfarlane, M. G. & Knight, B. C. J. G. (1941). *Biochem. J.* **35**, 884.
Macfarlane, R. G., Oakley, C. L. & Anderson, C. G. (1941). *J. Path. Bact.* **52**, 99.
Nagler, F. P. O. (1939). *Brit. J. exp. Path.* **20**, 473.
Nagler, F. P. O. (1945). *Aust. J. exp. Biol. med. Sci.* **23**, 59.
Oakley, C. L., Warrack, G. H. & Clarke, P. H. (1947). *J. gen. Microbiol.* **1**, 91.
Zamecnik, P. C., Brewster, L. E. & Lipmann, F. (1947). *J. exp. Med.* **85**, 381.

The Effect of Alcohols on the Hydrolysis of Sodium Phenolphthalein Diphosphate by Prostatic Extracts

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King (1943) synthesized several phosphoric esters which liberate chromogens after phosphatase hydrolysis. Among these were the calcium, barium and lead salts of phenolphthalein phosphate. He found, however, that these compounds were not suitable as a substrate for plasma phosphatase determinations owing to the slow rate of hydrolysis. Huggins & Talalay (1945) synthesized the sodium salt of phenolphthalein phosphate and considered it to be sufficiently rapidly hydrolyzed by phosphatase to constitute a suitable substrate for measuring phosphatase activity in plasma and other biological fluids. They described a method of measuring acid phosphatase activity using this substrate buffered at pH 5·4 and measuring colorimetrically the free phenolphthalein liberated. Whilst comparing this method with that of Gutman, Gutman & Robinson (1939), it was noticed that the addition of ethanol to the digest greatly increased the free phenolphthalein liberation.

EXPERIMENTAL

Preparation of enzyme. Human prostate was used as enzyme source. Partial purification was effected by the method of Kutscher & Worner (1936) slightly modified. The prostate was finely chopped, suspended in three times its own weight of distilled water, and autolyzed under toluene for 3 or 4 days. After centrifuging, the supernatant fluid was dialyzed against distilled water, and the protein precipitate filtered off. The preparation was freeze-dried and stored in the refrigerator. For use 6 mg. were dissolved in 50 ml. of distilled water.

Pentasodium phenolphthalein diphosphate was synthesized and acid-phosphatase activity estimated by the method of Huggins & Talalay (1945). Chloroform, however, was omitted from the substrate solution because irregular results were found in its presence.

Procedure. The phosphate liberated was measured as in the Bodansky (1933) method for the estimation of alkaline phosphatase. Simultaneous estimations of phenolphthalein and phosphate were carried out after digestion at 37° for 1 hr., both without any alcohol, and in the presence of ethanol, methanol, *n*-propyl and *iso*propyl alcohols and ethylene glycol.

The following buffered substrate was used: 1·216 g. sodium phenolphthalein phosphate, 11·7 g. sodium acetate (hydrated) and 0·79 ml. glacial acetic acid, dissolved in water and made up to 1 l. Each estimation was carried out in duplicate. Colorimeter tubes were set up in the water bath at 37° containing:

Tube (1) 2·5 ml. buffered substrate; 2·5 ml. distilled water; 0·5 ml. enzyme solution.

Tube (2) 2·5 ml. buffered substrate; 2·5 ml. diluted alcohol; 0·5 ml. enzyme solution.

1 ml. was immediately removed and discarded from (1), and 1 ml. from (2) was removed and run into a tube containing 5 ml. 12% (w/v) trichloroacetic acid and 4 ml. distilled water. This was centrifuged, and the initial phosphate determined by the amount of colour developed from 5 ml. of supernatant fluid. The tubes were then incubated for 1 hr., after which sufficient pure alcohol was added to (1) to give it the same concentration of alcohol as (2). The volume of (2) was then adjusted to that of (1) by adding alcohol of the appropriate strength. This dilution of the digest was allowed for in calculating the results. 1 ml. was then removed from (1) and (2) into tubes containing 5 ml. 12% (w/v) trichloroacetic acid and 4 ml. distilled water, centrifuged, and the supernatant fluid used to obtain the amount of free phosphate after digestion. To the remainder of the solution in the colorimeter tubes, glycine buffer and water were added, and the amount of free phenolphthalein liberated determined.

RESULTS

The effect of different concentrations of ethanol on the phenolphthalein liberated by the enzyme was determined. Approximately 15% (v/v) was found to show the greatest effect (Fig. 1). For the other alcohols, a concentration giving a marked increase in the amount of phenolphthalein liberated was chosen. The differences between the amounts of phenolphthalein and phosphate liberated with and without alcohol were measured, and the percentage increase or decrease calculated (Table 1). For each alcohol there is a large increase in the amount of free phenolphthalein liberated. As regards phosphate liberation it appears that methyl, ethyl and *n*-propyl alcohols, at the concentrations used, cause no increase in phosphate liberation, whereas *iso*-propyl alcohol produces a marked increase in the amount of phosphate liberated, and ethylene glycol a marked decrease.

DISCUSSION

Axelrod (1947) using an acid-optimum phosphatase prepared from orange juice, and monophenyl phosphate, *p*-nitrophenyl phosphate or sodium phenolphthalein phosphate as substrate, found that in the presence of certain alcohols, transference of phosphate from the substrate to the alcohol is catalyzed

by a newly described enzyme. Such a reaction would account for the results described in the present communication, assuming that there are two enzymes present, an acid-optimum phosphatase and a transphosphorylase which catalyze the following reactions:

(*a*) ϕ-Phosphate $\xrightarrow{\text{Phosphatase}}$ Phosphate + ϕ,

(*b*) ϕ-Phosphate + A $\xrightarrow{\text{Transphosphorylase}}$ A-phosphate + ϕ.

ϕ = phenolphthalein; A = alcohol.

Thus, in the presence of an alcohol, there is an increase in the liberation of free phenolphthalein, but no such effect on phosphate liberation.

In the case of ethylene glycol, the transphosphorylase presumably competes more effectively than the phosphatase for the phenolphthalein phosphate, whereas the case of *iso*propyl alcohol suggests that the alcohol phosphate is split again. Fig. 1 indicates that the transphosphorylase is inactivated by 20% (v/v) ethanol.

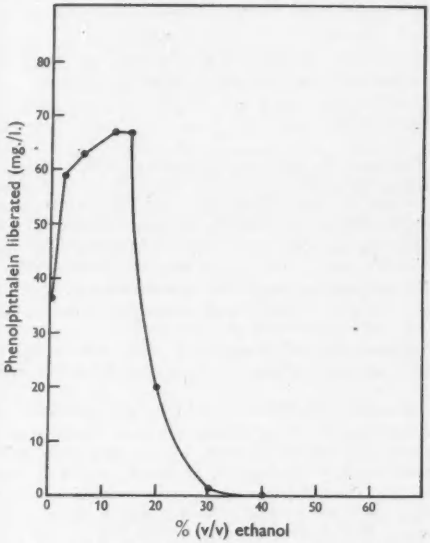

Fig. 1. The effect of different concentrations of ethanol on the production of free phenolphthalein by the action of prostatic phosphatase extract on pentasodium phenolphthalein diphosphate.

SUMMARY

1. The formation of free phenolphthalein from pentasodium phenolphthalein diphosphate by prostatic phosphatase preparations acting at pH 5·4 and 37° is increased by methyl, ethyl, *n*-propyl and *iso*propyl alcohols, and ethylene glycol.

2. The formation of free phosphate under the above conditions is apparently unaffected by methyl, ethyl, and *n*-propyl alcohols, increased by *iso*propyl alcohol and decreased by ethylene glycol.

3. An interpretation of these results in terms of transphosphorylation is suggested.

I should like to thank Dr N. R. Lawrie for his help during this work, and Dr M. Dixon for many valuable discussions.

Table 1. *The effect of alcohols upon the amounts of phenolphthalein and phosphate liberated from pentasodium phenolphthalein diphosphate, by incubation with a prostatic extract*

(ϕ = phenolphthalein. A = alcohol. Inorganic phosphate is expressed as mg. P/l.)

Alcohol (%, v/v)	ϕ liberated (mg./l.)		P liberated (mg./l.)		Increase (%)	
	With A	Without A	With A	Without A	ϕ	P
15% ethyl	75·8	45·0	18·6	19·4	+ 66	− 4
	94·5	58·5	16·6	15·6	+ 62	+ 6
	91·0	52·5	20·4	18·5	+ 74	+10
10% methyl	89·0	52·0	16·3	17·1	+ 71	− 5
	97·0	54·0	15·7	16·9	+ 79	− 7
	90·0	49·2	15·4	15·9	+ 83	− 3
5% *n*-propyl	86·5	56·0	20·9	19·7	+ 53	+ 6
	85·0	56·0	21·6	22·4	+ 52	− 7
	90·0	58·0	20·3	18·9	+ 55	+ 6
10% *iso*propyl	88·5	56·5	27·7	20·7	+ 57	+34
	83·0	51·5	26·5	17·7	+ 61	+50
	88·5	54·5	25·4	18·2	+ 62	+39
20% ethylene glycol	92·5	53·5	18·0	20·0	+ 73	−10
	89·5	41·2	11·8	19·4	+117	−39
	94·5	51·5	15·7	22·2	+ 84	−30

REFERENCES

Axelrod, B. (1947). *Fed. Proc.* **6**, 235.
Bodansky, A. (1933). *J. biol. Chem.* **99**, 197.
Gutman, A. B., Gutman, E. B. & Robinson, J. N. (1939). *J. Urol.* **42**, 602.
Huggins, C. & Talalay, P. (1945). *J. biol. Chem.* **159**, 399.
King, E. J. (1943). *J. Path. Bact.* **55**, 311.
Kutscher, W. & Worner, A. (1936). *Hoppe-Seyl. Z.* **239**, 109.

Reversibility of a Phosphoroclastic Reaction

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Utter, Lipmann & Werkman (1945) using C^{13} as a tracer showed reversibility of the phosphoroclastic reaction in *Escherichia coli* according to the equation:

$$CH_3COC^{13}OOH + H_3PO_4 \rightleftharpoons CH_3COOPO_3H_2 + HC^{13}OOH. \quad (1)$$

Esch. coli possesses an enzyme, hydrogenlyase, which reversibly performs the reaction:

$$HCOOH \rightleftharpoons H_2 + CO_2. \quad (2)$$

Reaction 1 occurring with reaction 2 involves carbon dioxide fixation.

Koepsell & Johnson (1942) obtained a cell-free extract from *Clostridium butylicum* which converts pyruvic acid into acetic acid, carbon dioxide and hydrogen, and requires phosphate for the reaction. Later Koepsell, Johnson & Meek (1944) found that inorganic phosphate is taken up and labile phosphate appears. The overall reaction may be illustrated:

$$CH_3COCOOH + H_3PO_4 \rightarrow CH_3COOPO_3H_2 + CO_2 + H_2. \quad (3)$$

The question arises whether formic acid occurs as an intermediate (Osburn, Brown & Werkman (1937) and Stiles, Peterson & Fred (1929)). This communication deals with the reversibility of reaction 3, and evidence is presented that formic acid is not an intermediate. Reaction 3 appears to differ fundamentally from the phosphoroclastic reaction of *Esch. coli*.

METHODS

Cl. butylicum (Beijerinck) Donker was used in this investigation. The organism was grown in a medium of 1% glucose, 0·4% yeast extract, 0·4% peptone, 0·1% beef extract, 0·05% reduced iron, 10% tap water and 1·25% K_2HPO_4 and adjusted to pH 7 with HCl. The glucose and K_2HPO_4 were sterilized separately and added aseptically to the medium. The medium was inoculated with 100 ml. of an 18 hr. culture and incubated at 37° for 18–24 hr. The medium was strained through cheese cloth to remove most of the iron. The cells were harvested with a Sharples centrifuge. The yield varied from 1·5 to 2·5 g./l. of medium. A cell-free extract was prepared from these cells by the method of Utter, Kalnitsky & Werkman (1946). Experiments with this extract were conducted in 125 ml. manometric flasks with total volumes indicated in the tables.

Reversibility was determined by incubating pyruvic acid with a properly buffered enzyme preparation in the presence of one or the other of the products containing an excess of C^{13}. The reaction was stopped short of completion and the residual pyruvic acid was recovered, degraded and analyzed for excess C^{13}.

Separation of the pyruvic acid from the acetic acid was accomplished by two methods: (1) steam distillation as carried out by Utter *et al.* (1945), and (2) ether extraction of the acetic acid in the presence of $NaHSO_3$ which forms an ether-insoluble complex with pyruvic acid. The bisulphite complex was decomposed by boiling and the pyruvic acid removed by ether extraction.

When only the carboxyl carbon of pyruvic acid was desired for isotopic analysis, it was obtained by oxidation with ceric sulphate, which oxidizes pyruvic acid to acetic acid and CO_2. The acetic acid was separated by steam distillation and oxidized to CO_2 with $K_2S_2O_8$. For the isotopic analysis of each carbon atom in the pyruvic acid the latter was degraded first by yeast Lebedev juice which decarboxylates pyruvic acid to acetaldehyde and CO_2. The acetaldehyde was treated with NaOI, when the methyl group formed iodoform and the aldehyde group formic acid. The iodoform was oxidized by dry combustion to CO_2 and the formic acid was oxidized to CO_2 by the method of Osburn & Werkman (1932).

$CH_3C^{13}OOH$ was prepared by means of the Grignard reaction. $HC^{13}OOH$ was prepared by the method of Utter *et al.* (1945). $C^{13}O_2$ was added as $NaHC^{13}O_3$.

RESULTS

Fixation of $C^{13}O_2$ *in the carboxyl group of pyruvic acid.* When the buffered enzyme preparation was incubated with sodium pyruvate and $NaHC^{13}O_3$ under the conditions of Table 1, the residual pyruvate contained C^{13} only in the carboxyl group. The

Table 1. *Fixation of* $C^{13}O_2$ *in pyruvic acid by enzyme extract from* Clostridium butylicum

(Reaction mixture: 375 mg. lyophilized *Cl. butylicum* extract, 1·6 mmol. $NaHC^{13}O_3$ containing 5·43% (atom) excess C^{13}, 2·0 mmol. pyruvate, 1·08 mmol. phosphate, pH 6·8; total volume, 30 ml. Time, 7 hr.; temp. 30°. Atmosphere, 100% N_2.)

Pyruvic acid utilized (mmol.)	Excess of C^{13} in (atom %)		
	Residual CO_2	CH_3CO— of pyruvic acid	—COOH of pyruvic acid
0·66	1·96	0·00	1·98

C^{13} contents of the carboxyl carbon and the residual CO_2 indicate that an equilibrium was established. There was no excess C^{13} in the CH_3CO— group.

Effect of hydrogen on fixation of $C^{13}O_2$. Lipmann & Tuttle (1945) observed that molecular hydrogen inhibited the breakdown of pyruvic acid by the enzyme extract of *Cl. butylicum*. It was of interest to determine the effect of hydrogen on the fixation of $C^{13}O_2$ under conditions similar to those of the previous experiment except that the atmosphere consisted of 50% hydrogen and 50% nitrogen (Table 2). Fixation was substantially greater in the presence of hydrogen, although the amount of pyruvate dissimilated was less than in the presence of 100% nitrogen. Apparently the point of equilibrium in the presence of hydrogen is farther to the left, resulting in greater fixation.

Table 2. *Effect of hydrogen on fixation of* $C^{13}O_2$ *by* Clostridium butylicum

(Reaction mixture: 15 ml. enzyme extract, 1·6 mmol. $NaHC^{13}O_3$ (5·43% excess C^{13}), 2·0 mmol. pyruvate, 1·08 mmol. phosphate, pH 6·8; total volume, 30 ml. Time, 3 hr.; temp. 30°. Degradation by ceric sulphate.)

Atmosphere	Pyruvic acid utilized (mmol.)	Excess of C^{13} in (atom %)		
		Residual CO_2	CH_3CO— of pyruvic acid	—COOH of pyruvic acid
100% N_2	1·19	1·90	0·00	1·59
50% N_2 + 50% H_2	0·78	1·80	0·00	1·75

Exchange between $CH_3C^{13}OOH$ *and* $CH_3COCOOH$. If equation (3) is reversible, then before acetic acid could participate in it, it would first need to be phosphorylated to acetyl phosphate. Results of Table 3 show the results of adding $CH_3C^{13}OOH$ without an added energy-rich source of phosphate. In order to ensure adequate separation of the pyruvic acid from the $CH_3C^{13}OOH$, both the bisulphite-ether extraction method and the double steam distillation were employed on each sample. The pyruvic acid was degraded by the yeast carboxylase and by the ceric sulphate methods, and essentially the same C^{13} content was obtained by both methods. Results in Table 3 were obtained by the ceric sulphate method. The C^{13} content of the —CO— group was calculated from the content of the CH_3CO— group. The average values obtained with repeated experiments showed the same magnitude of fixation. The calculated value of C^{13} for the —CO— group is nearly in equilibrium with the residual $CH_3C^{13}OOH$. Results of an experiment carried out with whole cells (when possibly greater exchange might occur) did not significantly differ from those obtained with the enzyme extract.

Table 3. *Exchange between* $CH_3C^{13}OOH$ *and* $CH_3COCOOH$

(Reaction mixture: 6 ml. enzyme extract from *Cl. butylicum*, 1·1 mmol. $CH_3C^{13}OONa$ (4·65% excess C^{13}), 2·0 mmol. pyruvate, 1·08 mmol. phosphate buffer, pH 6·8; total volume, 30 ml. Time, 60 min.; temp. 30°. Atmosphere, 95% H_2 + 5% CO_2. Pyruvate dissimilated, 1·39 mmol.)

Reaction product examined	Excess C^{13} (atom %)
Residual CH_3COOH	0·11
Pyruvate:	
—COOH	0·00
CH_3CO—	0·04
—CO—	0·08 (calc.)

Effect of adenosine triphosphate (ATP) and acetyl phosphate on the exchange of $CH_3C^{13}OOH$. Utter *et al.* (1945) working with cell-free extracts of *Esch. coli* found that significant fixation of $CH_3C^{13}OOH$ in pyruvate occurred only in the presence of adenosine triphosphate. The high-energy phosphate was required apparently for the phosphorylation of the acetic acid. Table 4, Exp. 1, records the results of a typical experiment carried out in the presence of *ATP* and $CH_3C^{13}OOH$. Exp. 2 contained acetyl phosphate and adenylic acid in addition to the $CH_3C^{13}OOH$. In the presence of acetyl phosphate, adenylic acid and $CH_3C^{13}OOH$ (Exp. 2) the C^{13} content of the CH_3CO— group of the pyruvate was significantly higher than when $CH_3C^{13}OOH$ was used alone. The C^{13} content is also greater in the presence of *ATP* (Exp. 1); however, it is not as high

Table 4. *Effect of ATP and acetyl phosphate on* $CH_3C^{13}OOH$ *fixation*

(Each experimental mixture contained 5 ml. *Cl. butylicum* extract, 2 mmol. pyruvate, 1·06 mmol. $CH_3C^{13}OONa$ (4·65% excess C^{13}), 0·1 mmol. $MgCl_2$, and 1·08 mmol. phosphate buffer, pH 6·8. In addition, Exp. 1 contained 34 mg. adenylic acid and 75 mg. acetyl phosphate. Exp. 2 contained 195 mg. *ATP*. Atmosphere, 95% H_2 and 5% CO_2. Time, 1·5 hr.; temp. 30°.)

Grouping of pyruvate molecule which was estimated	Exp. 1, with adenylic acid + acetyl phosphate		Exp. 2, with adenosine triphosphate	
	Quantity (mmol.)	Excess C^{13} (atom %)	Quantity (mmol.)	Excess C^{13} (atom %)
—COOH	0·64	0·00	0·36	0·00
CH_3CO—	1·18	0·12	2·20	0·06
—CO—	—	0·24 (calc.)	—	0·12 (calc.)

as in Exp. 2. Assuming that the CH_3 group of the pyruvate contains no C^{13}, as indicated by degradation by yeast carboxylase and the iodoform reaction, calculation of the C^{13} content of the —CO— group gives values which are significant. The presence of the C^{13} in the —CO— group is expected from the fixation of $CH_3C^{13}OOH$.

Exchange between $HC^{13}OOH$ *and* $CH_3COCOOH$. Since it was questionable whether formic acid was an intermediate in the reaction, an experiment was carried out with pyruvic acid and $HC^{13}OOH$. If the reaction is similar to that in *Esch. coli*, exchange should occur with pyruvic acid. Since the occurrence of formic acid in the dissimilation of pyruvic acid by *Cl. butylicum* was in question, $HC^{13}OOH$ was used in an experiment with pyruvic acid. If the reaction with *Cl. butylicum* is similar to that of *Esch. coli*, C^{13} should be found in the residual pyruvic acid. The results (Table 5) show conclusively that formate

Table 5. *Absence of exchange between* $HC^{13}OOH$ *and* $CH_3COCOOH$

(Reaction mixture: 8 ml. *Cl. butylicum* extract, 2·0 mmol. phosphate buffer, pH 6·8; 1 mmol. $HC^{13}OOH$ (2·31% excess C^{13}), 2·0 mmol. pyruvate, 25 mg. *ATP*; total vol., 37 ml. Time, 60 min.; temp. 30°. Atmosphere, 50% H_2 and 50% N_2. Pyruvate dissimilated, 0·88 mmol.)

Reaction product examined	Excess C^{13} (atom %)
Residual $NaHCO_3$	0·00
Pyruvate:	
—COOH	0·00
CH_3CO—	0·00
Formate	2·32

does not exchange with pyruvate, and from the C^{13} content of the residual $NaHCO_3$ it is evident that the formate does not break down to H_2 and CO_2.

DISCUSSION

The phosphoroclastic reaction in *Cl. butylicum* has been shown to be reversible. Formic acid was not found to be an intermediate. This appears to be the first demonstration of C_2 and C_1 addition where carbon dioxide, *per se*, is the C_1 compound fixed. Such a fixation clarifies the data of Brown, Wood & Werkman (1944) who found an excess of C^{13} in the carboxyl group of lactic acid formed by *Cl. butylicum* from glucose in the presence of $C^{13}O_2$. Since no appreciable amounts of C_4 compounds were detected, no significant fixation of the C_3+C_1 type could have occurred.

Rigorous proof that acetyl phosphate is an intermediate in the reaction remains to be demonstrated; however, the presence of an energy-rich phosphate source together with $CH_3C^{13}OOH$ does enhance the reversibility of the reaction. In the phosphoroclastic reaction of *Esch. coli*, reduction of carbon dioxide to formic acid occurs prior to the C_2 and C_1 addition. With *Cl. butylicum* there is either a simultaneous reduction with fixation, or fixation occurs with subsequent reduction. It is difficult to visualize the latter possibility with acetic acid or acetyl phosphate as the C_2 compound concerned in the fixation. The possibility remains that some C_1 reduced compound other than formic acid is a component of the system.

The observation that an atmosphere of hydrogen enhanced fixation of carbon dioxide in pyruvic acid strengthens the evidence for reversibility of the reaction.

SUMMARY

1. The conversion of pyruvic acid into acetic acid, carbon dioxide and hydrogen by *Clostridium butylicum* which requires phosphate has been demonstrated by the use of $NaHC^{13}O_3$ to be reversible.

2. It is shown that molecular hydrogen enhances fixation of carbon dioxide in this reaction.

3. Formic acid is not an intermediate and it is suggested that acetyl phosphate might be a component of the system, although no rigorous proof for this hypothesis has as yet been obtained.

REFERENCES

Brown, R. W., Wood, H. G. & Werkman, C. H. (1944). *Arch. Biochem.* **5**, 423.

Koepsell, H. J. & Johnson, M. J. (1942). *J. biol. Chem.* **145**, 379.

Koepsell, H. J., Johnson, M. J. & Meek, J. S. (1944). *J. biol. Chem.* **154**, 535.

Lipmann, F. & Tuttle, L. C. (1945). *J. biol. Chem.* **158**, 505.

Osburn, O. L., Brown, R. W. & Werkman, C. H. (1937). *J. biol. Chem.* **121**, 685.

Osburn, O. L. & Werkman, C. H. (1932). *Industr. Engng Chem.* (Anal. ed.), **4**, 421.

Stiles, H. R., Peterson, W. H. & Fred, E. B. (1929). *J. biol. Chem.* **84**, 437.

Utter, M. F., Kalnitsky, G. & Werkman, C. H. (1946). *Arch. Biochem.* **9**, 285.

Utter, M. F., Lipmann, F. & Werkman, C. H. (1945). *J. biol. Chem.* **158**, 521.

The Infrared Examination of Compounds Related to Ascorbic Acid

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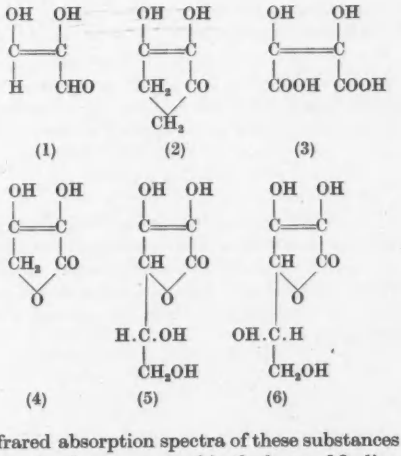
It is now realized that infrared absorption spectra can frequently be used with marked success in the differentiation of organic substances for the analysis of mixtures and for structural diagnosis. The present paper indicates briefly how it may be applied in one problem of interest in biochemistry.

Recent studies on vitamin C in plant materials have shown that its occurrence and/or functions may be better understood by reference to other closely related dienols, such as reductone (1), reductic acid (2), hydroxymaleic acid (3) and hydroxytetronic acid (4). These dienols, although possessing no anti-scorbutic activity, all react with the indophenol dye normally used to estimate ascorbic acid (5). The first three, which are known to occur naturally, can be differentiated from ascorbic acid by various modifications of Lugg's (1942) formaldehyde method (cf. Mapson, 1943 *a*, *b*; Wokes, Organ & Jacoby, 1943). Hydroxytetronic acid cannot be so differentiated, neither can *iso*ascorbic acid (6), which is known to occur in certain commercial fruit juices. Moreover, the formaldehyde method does not differentiate between 1, 2, 3 and 4.



The infrared absorption spectra of these substances have therefore been measured in the hope of finding characteristic differences which may be used for analysis. The results given below show that such distinguishing features occur, and these may not only enable the analyses to be carried out, but may also provide a method which may help in following the course of plant processes.

EXPERIMENTAL

The spectra were measured between 2–14 μ. with an automatically recording single beam spectrometer, with rock salt prism (Whiffen & Thompson, 1945). A few measurements were also made in the region 5–7 μ. with a double beam spectrometer having a prism of calcium fluoride.

The compounds were obtained from Roche Products Ltd., except for dihydroxymaleic acid, which was a laboratory specimen. No impurities could be detected by their ultra-violet absorption spectra. A specially purified sample of reductic acid supplied by Prof. Reichstein was also used. The spectra of the solid powders were measured by grinding to a paste with a little paraffin in the standard manner. A satisfactory spectrum can be obtained with a few mg. of material. The paste was pressed between two small rock salt plates. Measurement of the spectrum of the solids had the advantage over solutions that the whole spectrum could be surveyed without serious disturbance by absorption bands of the solvent. For analytical work, it would be necessary to work with dilute solutions in some solvent chosen so as to have clear transmission in the region of the key absorption bands concerned. Over small regions of the spectrum shown in Fig. 1, paraffin has its own absorption bands, which may obscure those of the substance, but these are regions where the vibrations due to C—H bonds occur and it is certain that, from an analytical standpoint, they are unimportant. These spectral regions are marked *N* in Fig. 1.

RESULTS AND DISCUSSION

Fig. 1 shows the absorption spectra of the six compounds. The positions of the bands are shown on a wave-length scale (μ.), and also on a frequency scale, the customary units of reciprocal wave lengths, or waves/cm. (cm.$^{-1}$) being used. It is seen that, whilst in several cases there are absorption bands at the same positions, each substance has a different spectrum as a whole. Each compound has a band near 1650 cm.$^{-1}$, presumably due to the stretching vibration of the C═C bond. Some of the bands in the region 1100–1250 cm.$^{-1}$ may be connected with bending vibrations of the hydroxyl groups. The three compounds having a lactone ring show a band at about 1750 cm.$^{-1}$, which has previously been found common to many lactones (Richards & Thompson, 1947). In the region 2400–3600 there is interesting and varied absorption due to the stretching vibration of bonded-hydroxyl groups. It is noteworthy that the different compounds show

marked differences in this respect, as has also been found in measurements on a series of sugars and their derivatives (Trotter & Thompson, 1947).

The spectra are unfortunately so complex that the analysis of a mixture of compounds 1, 2, 3, 4 and 5, if all were present together, would be very difficult. However, if only two or three components were present, it should be possible. For example, reductone could be determined in ascorbic acid by its band at 923 cm.$^{-1}$, or at 1580 cm.$^{-1}$, or ascorbic acid in reductone by its bands at 1030, 1120 or 1755 cm.$^{-1}$. Reductone could be differentiated from reductic acid by its bands at 777 or 923 cm.$^{-1}$, and reductic acid in reductone by its band at 1126 cm.$^{-1}$. A key band for dihydroxymaleic acid may be that at 950 cm.$^{-1}$. Differentiation of hydroxytetronic acid from reductic acid could be carried out with the bands between 750–900 cm.$^{-1}$. For the differentiation of ascorbic acid and *iso*ascorbic acid the bands at 991 and 941 cm.$^{-1}$ respectively seem very suitable.

As with all other analyses in the infrared, the sensitivity and accuracy will depend upon the precise mixtures to be analyzed, and will probably vary from case to case. We propose to use the method in studying the growth processes of certain plants, but it seems desirable to draw attention now to the possibilities of this type of measurement.

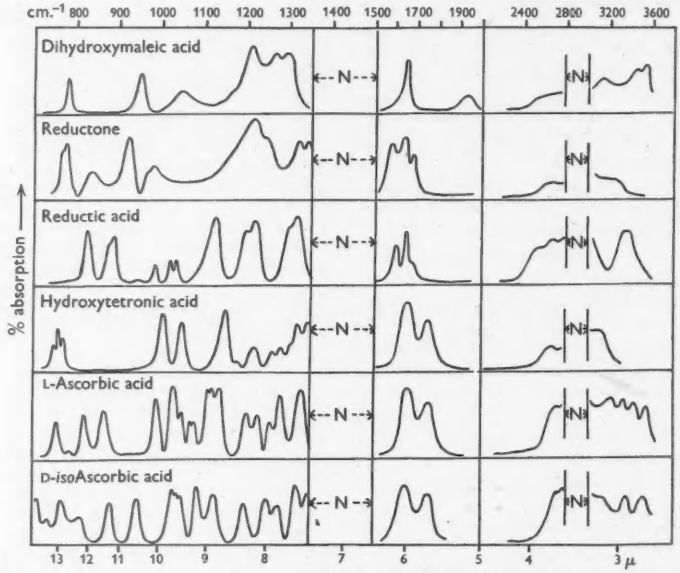

cm.$^{-1}$ 800 900 1000 1100 1200 1300 1400 1500 1700 1900 2400 2800 3200 3600
Dihydroxymaleic acid
Reductone
Reductic acid
Hydroxytetronic acid
L-Ascorbic acid
D-*iso*Ascorbic acid
N
% absorption
13 12 11 10 9 8 7 6 5 4 3 μ

Fig. 1. Infrared absorption curves of compounds related to ascorbic acid. Region marked *N* obscured by bands of paraffin. Percentage absorption covers the range 100%.

SUMMARY

The infrared absorption spectra of some compounds related to ascorbic acid have been measured between 2–14 μ. There are differences between the spectra which should make analyses of mixtures possible and more satisfactory than by existing chemical methods.

REFERENCES

Lugg, J. W. H. (1942). *Aust. J. exp. Biol. med. Sci.* **20**, 273.

Mapson, L. W. (1943*a*). *Nature, Lond.*, **152**, 13.

Mapson, L. W. (1943*b*). *J. Soc. chem. Ind., Lond.*, **62**, 223.

Melville, R., Wokes, F. & Organ, J. G. (1943). *Nature, Lond.*, **152**, 447.

Richards, R. E. & Thompson, H. W. (1947). Unpublished reports on penicillin research.

Trotter, I. F. & Thompson, H. W. (1947). Unpublished data.

Whiffen, D. H. & Thompson, H. W. (1945). *J. chem. Soc.* p. 268.

Wokes, F., Organ, J. G. & Jacoby, F. C. (1943). *J. Soc. chem. Ind., Lond.*, **62**, 232.

The *in vitro* Synthesis of Glycogen in the Diaphragms of Normal and Alloxan-diabetic Rats

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Gemmill (1940, 1941) and Gemmill & Hamann (1941), confirmed by Hechter, Levine & Soskin (1941) and by Stadie (1944), showed that surviving rat diaphragm can synthesize glycogen from added glucose. The rate of this synthesis may be increased by addition of insulin *in vitro*. Following these findings the *in vitro* synthesis of glycogen from glucose as well as glycogenolysis by muscle were further investigated. It was proposed to examine (*a*) the influence of the composition of the medium, and especially the effect of potassium, on these reactions, (*b*) the influence of adrenaline, (*c*) the effect of insulin in normal animals, and (*d*) the synthesis of glycogen and glycogenolysis in diaphragms from alloxan-diabetic rats.

Cross & Holmes (1937) and Ostern, Herbert & Holmes (1939) found that liver slices can synthesize glycogen *in vitro* from added glucose. Hastings & Buchanan (1942) demonstrated that in rat-liver slices this synthesis takes place only in a potassium-rich, sodium-free medium, corresponding in ionic composition to intracellular fluid. In a medium resembling extracellular fluid no synthesis of glycogen takes place. Saxton & Miller (1944) showed that even under optimal conditions this synthesis of glycogen depends on the time interval which elapses between the death of the animal and the beginning of the experiment *in vitro*. On the basis of these experiments the synthesis of glycogen and glycogenolysis in rat-liver slices were studied *in vitro*, and were compared with the same processes in the diaphragm.

EXPERIMENTAL

Methods

Preparation of the diaphragm. Male albino rats of laboratory stock weighing 80–120 g. were used. The animals were anaesthetized with nembutal (pentobarbital) and bled. The diaphragm was quickly excised and two equal portions were weighed. In control experiments with both halves incubated under identical conditions, we observed the same deviations as were reported by Gemmill (1940). Dividing the diaphragm of older rats into three or more equal parts proved to be unreliable.

Media for incubation. (*a*) *Buffered salt solutions.* The diaphragm samples were incubated according to Gemmill (1940) at 38° in a medium described by Gey & Gey (1936) and modified by Gemmill (1940). Its composition was: 0·7% NaCl, 0·037% KCl, 0·227% $NaHCO_3$, 0·017% $CaCl_2$, 0·015% Na_2HPO_4, and 0·003% KH_2PO_4 (all % w/v). This medium was similar to the solution described by Van Dyke & Hastings (1928), and resembled body fluid in its ionic composition. It is referred to here as 'extracellular solution'. In our experiments modifications of this solution by addition or removal of an ion were compensated for by corresponding changes in NaCl content. With a modification of this solution, containing only 0·74% NaCl, 0·015% Na_2HPO_4 and 0·003% NaH_2PO_4, we obtained optimal synthesis of glycogen by rat diaphragm *in vitro*. This solution is called 'phosphate buffered saline'. The glucose content of the media was usually 100 mg./100 ml., the concentration most favourable according to Hechter *et al.* (1941) for the demonstration of the insulin effect.

(*b*) *Serum.* For 'normal serum' pooled human serum was generally used, its glucose content being adjusted to 100 mg./100 ml. No differences were found with respect to synthesis of glycogen between sera of various animals, and fresh rabbit or rat sera were also used. When comparing synthesis in normal and diabetic serum, only freshly prepared rat serum was used, and the glucose content of the normal serum was adjusted to that of the diabetic serum. In another series of experiments the synthesis of glycogen in normal serum was investigated at a glucose concentration of 800 mg./100 ml., this being the mean serum glucose content in a series of alloxan-diabetic rats. The diaphragms of normal and alloxan-diabetic rats are termed 'normal diaphragm' and 'diabetic diaphragm', respectively, and the sera of these animals are similarly designated.

Bicarbonate-containing buffer media and sera were aerated with a gas mixture containing 95% O_2 and 5% CO_2, the bicarbonate-free phosphate-buffered saline with O_2. All solutions proved to be sufficiently buffered, and the experiments were carried out at a pH of approximately 7·4. No significant changes in pH were found after 3 hr. incubation with diaphragm even in unbuffered 0·9% NaCl. A concentrate of rat serum of approximately double strength was prepared according to Kanner (1946) by means of purified gelatin.

Measurement of glycogen synthesis. As measure of glycogen synthesis the difference in glycogen content between one test sample incubated for 6 min. and the other for 3 hr. is used. This value is termed 'glycogen synthesis value'. The influence of various factors, such as ionic composition and additions of drugs, was measured in most of our experiments by the difference in the glycogen content of two samples after 3 hr. incubation, one sample being incubated in the usual medium and the other in medium modified in the appropriate manner. This value is called 'glycogen

difference value'. The values are expressed in mg. increase/100 mg. diaphragm. The 'difference value' is also expressed as the percentage increase or decrease in glycogen.

The incubation of liver slices. Experiments on liver slices were carried out in the 'intracellular solution' described by Hastings & Buchanan (1942). The incubation time in these experiments was 4 hr., and glucose concentrations used were 0·25, 0·5 and 1%.

Experiments on glycogenolysis. These experiments were carried out in the extra- and intracellular solutions mentioned above without the addition of glucose. Serum was freed of sugar by incubation with yeast for 30 min. Glycogenolysis is expressed as the decrease in the glycogen content after 3 hr. incubation. Since the rate of glycogenolysis is dependent on the initial glycogen content, the values are given as the percentage decrease in glycogen as well as in mg./100 mg. tissue.

Respiration measurements. Determinations of oxygen uptake and respiratory quotient were carried out by Warburg's direct method (Dixon, 1943) in phosphate-Ringer. Measurements were made over periods varying from 2 to 3 hr. and the average value for 1 hr. was recorded.

Chemical procedures. Glycogen determinations in the diaphragm were carried out by a micromodification of Pflüger's method, employing liver slices according to Good, Kramer & Somogyi (1933). Glucose was estimated according to Shaffer, Hartmann & Somogyi (Somogyi, 1937).

Production of diabetes. Diabetes was induced by intravenous injection of 6–8 mg. of alloxan per 100 g. rat. Animals in various stages of diabetes were used, varying from those with severe coma to those with latent diabetes which exhibited glycosuria only on carbohydrate-rich food. Ordinary insulin (Squibb) was used, 2 units usually being added to 3 ml. of medium; this quantity corresponds approximately to the mean dose used by Gemmill. The ergotamine tartrate employed was Gynergen (Sandoz).

RESULTS

(1) *Influence of the composition of the medium, and especially of the potassium content on glycogen synthesis and glycogenolysis*

Removal of potassium from the 'extracellular solution' resulted in an increased synthesis of glycogen from glucose in rat diaphragm. Not only was the presence of calcium unnecessary for the synthesis of glycogen, but in a calcium-free medium the synthesis was even slightly improved, the increase being 20% ± 8 in 13 experiments. NH_4^+ had the same inhibiting effect as potassium. Optimal synthesis was observed in our experiments in phosphate-buffered saline. Two- or threefold increase in the potassium content of the 'extracellular solution' nearly always resulted in complete inhibition of glycogen synthesis (Table 1). In the 'intracellular' potassium-rich, sodium-free medium, described by Hastings & Buchanan (1942), intensive glycogenolysis occurred instead of synthesis, and was already manifest after 15 min. On the other hand, as demonstrated by these authors, glycogen synthesis in rat-liver slices takes place only in the intracellular medium. If in these experiments with liver slices part of the KCl is replaced by NaCl, glycogen synthesis is inhibited, but still demonstrable. With 'intracellular solution' glycogen can still be synthesized by liver *brei*, but only at the high glucose concentrations of 1·5–2%. In 13 experiments a mean increase of 0·63 mg. ± 0·19/g. liver *brei* was observed. Under no conditions could synthesis of glycogen be obtained in diaphragm *brei*. Contrary to our findings in the diaphragm, Ostern *et al.* (1939), working with liver slices, noted that the removal of calcium resulted in inhibition of glycogen synthesis.

Glycogen synthesis in the diaphragm was optimal at pH 7·4, and decreased by 13% ± 7 at pH 6·8. If serum was used as medium the synthesis of glycogen in the diaphragm was of the same order of magnitude as in phosphate-buffered saline (Table 1). Although the potassium content of serum is the same as that of the 'extracellular solution', no inhibitory effect could be observed with serum. Two- or threefold increase of the serum potassium, however, did result in a decreased synthesis of glycogen. Replacing one third to one fifth of the 'extracellular solution' by serum improved the synthesis of glycogen. No increase in synthesis could be induced by addition of serum to phosphate-buffered saline.

Glycogenolysis was of similar magnitude in phosphate-buffered saline and in serum. Much more intense glycogenolysis was observed with the 'extracellular solution' (Table 2).

Table 1. *Synthesis of glycogen in normal rat diaphragm in various media compared with synthesis in the 'extracellular solution'*

No. of exp.	'Extracellular solution', compared with	Glycogen difference (mg./100 mg. tissue)	Glycogen difference Increase or decrease (%)
9	'Extracellular', potassium-free	+0·065±0·013	+48±13
6	'Extracellular' +0·01–0·015 M-KCl	−0·046±0·020	−15± 6
10	Serum	+0·057±0·018	+56±25
6	'Extracellular' +0·2–0·33 vol. of serum	+0·089±0·018	+28± 6
12	Serum +0·01–0·015 M-KCl	−0·046±0·018	−13± 6
13	Phosphate saline	+0·120±0·027	+87±25

Table 2. *The influence of different media on glycogenolysis in rat diaphragm*

Medium	No. of exp.	Decrease in glycogen (mg./100 mg. tissue)	(% decrease)
'Extracellular'	10	−0·126±0·028	−48±2
Phosphate saline	7	−0·049±0·017	−17±4·5
Serum	11	−0·068±0·024	−25±4

(2) *The influence of adrenaline*

The addition of adrenaline hydrochloride at a concentration of 0·33 μg./ml. regularly decreased glycogen synthesis. At concentrations of 0·033 μg./ml. the effect was irregular and at 0·0033 μg./ml. it disappeared. Inhibition could already be observed after 1 hr. incubation. Ephedrine at a concentration of 3·3 μg./ml. had the same effect as adrenaline, while tyramine at the same concentration did not change the rate of glycogen synthesis. The effect of adrenaline was not dependent on the presence of potassium. Ergotamine tartrate (8·3–10 μg./ml.) abolished the inhibitory effect of adrenaline on glycogen synthesis. Since ergotamine alone inhibits the synthesis of glycogen it must be assumed that its action under the conditions of this experiment is due to its antagonizing effect on adrenaline. Insulin weakened the effect of adrenaline in 8 out of 13 experiments (Table 3).

Table 3. *The influence of adrenaline and the antagonizing effect of ergotamine tartrate on synthesis of glycogen in diaphragm*

No. of exp.	Samples incubated	Glycogen difference (mg./100 mg. tissue)	Increase or decrease (%)
8	With and without adrenaline	−0·085±0·027	−44±7
8	With adrenaline, with and without ergotamine	+0·060±0·012	+31±5·5
3	With and without ergotamine	−0·060±0·014	+16±4

The effect of adrenaline on glycogenolysis is of the same order of magnitude as its effect on glycogen synthesis. Adrenaline at a concentration of 0·33 μg./ml. increased glycogenolysis by 47% ± 7 (6 experiments). Cross & Holmes (1937), working with liver slices, first demonstrated the inhibition *in vitro* of the synthesis of glycogen by adrenaline, albeit only at a concentration of 50 μg./ml. Bendall & Lehmann (1941) produced a twofold effect of adrenaline at a concentration of 0·45 μg./ml. on the synthesis of glycogen in liver slices: an initial reduction and a subsequent increase. No clear-cut effect of insulin on glycogenolysis produced by adrenaline could be observed.

(3) *The influence of insulin on glycogen synthesis and glycogenolysis*

The experiments of Gemmill (1940) on the influence of insulin on the synthesis of glycogen, in the solution used by him, were confirmed, and the same effect was also demonstrated in phosphate-buffered saline. In serum, however, no additional increase of synthesis could be induced by insulin (Table 5). No influence of insulin on glycogenolysis could be observed, either in the 'extracellular solution' or in phosphate-buffered saline.

(4) *Glycogen synthesis and glycogenolysis in the diaphragm of alloxan-diabetic rats: the influence of insulin*

In the 'extracellular solution' the synthesis of glycogen by diabetic diaphragm was certainly not less than that of normal diaphragm and may have been somewhat higher. In phosphate-buffered saline no difference was found between glycogen synthesis by normal and diabetic diaphragm. In serum the synthesis by the diabetic diaphragm was somewhat lower but not to a significant degree (Table 4). Glycogenolysis in the 'extracellular solution' was of the same order of magnitude in diabetic and normal diaphragms.

The addition of insulin to the 'extracellular solution' resulted in synthesis values of 0·130 mg./100 mg. diaphragm for the diabetic and 0·150 for the normal rat, synthesis values without insulin being 0·079 and 0·055, respectively. In phosphate-buffered saline the effect of insulin was markedly lower on the diabetic than on the normal diaphragm (Table 5).

Table 4. *Synthesis of glycogen in diaphragm of alloxan-diabetic and normal rats in different media*

Medium	Alloxan-diabetic rats: No. of exp.	Alloxan-diabetic rats: Synthesis (mg./100 mg. tissue)	Normal rats: No. of exp.	Normal rats: Synthesis (mg./100 mg. tissue)
'Extracellular'	23	0·079±0·015	18	0·055±0·013
Phosphate saline	13	0·080±0·017	14	0·109±0·020
Pooled normal serum	31	0·057±0·012	29	0·084±0·012

Table 5. *The influence of insulin on synthesis of glycogen in diaphragm of alloxan-diabetic and normal rats*

Medium	Alloxan-diabetic rats: No. of exp.	Alloxan-diabetic rats: Glycogen difference (mg./100 mg. tissue)	Normal rats: No. of exp.	Normal rats: Glycogen difference (mg./100 mg. tissue)
Phosphate saline	18	+0·044±0·037	20	+0·144±0·030
Normal pooled serum	22	+0·059±0·015	20	−0·032±0·022

In a further series of experiments the synthesis values of normal and of diabetic diaphragms, incubated in diabetic and in normal serum (glucose concentration 0·8%), were determined. The synthesis of glycogen in diabetic diaphragm was significantly diminished in diabetic serum, whereas in normal serum normal synthesis values were obtained. The synthesis of glycogen in normal diaphragm was markedly depressed in diabetic serum.

(5) *Respiration of normal and diabetic diaphragms**

In phosphate-Ringer solution, with and without glucose, the respiration of diabetic diaphragm was markedly higher than that of normal diaphragm. This result requires an explanation. Since glycogen synthesis differed in serum from that in phosphate-buffered saline, respiration experiments were carried out in normal and diabetic serum. In normal serum

Table 6. *Synthesis of glycogen in alloxan-diabetic and normal diaphragm incubated with alloxan-diabetic and normal serum and the effect of insulin*

Diaphragm	Serum	No. of exp.	Insulin	Synthesis (mg./100 mg. tissue)	No. of exp.	Difference (mg./100 mg. tissue)
Diabetic	Diabetic	18	−	0·122±0·022		
Normal	Normal	20	+	0·215±0·020		
Diabetic	Normal	12	−	0·204±0·036		
Normal	Diabetic	15	−	0·151±0·021†		
Diabetic	Diabetic	9	+	0·410±0·052	7	+0·171±0·040
Diabetic acidotic	Diabetic	6	+	0·135±0·027		
Diabetic	Diabetic, insulin-treated *in vivo*	7	−	0·290±0·050		
Diabetic	Diabetic (1/2) + normal serum concentrate (1/2)	7	−	0·201±0·017		
Normal	Normal	10	+	0·373±0·056	10	+0·076±0·046

† One experiment exhibited a distinctly higher synthesis value of 0·616 and is not included in this table.

Addition of insulin to diabetic serum produced a marked elevation of the synthesis value in diabetic diaphragm. Since the usual dose of 2 units of insulin was rather high, some experiments were carried out with one-tenth of this dose, which was found to be still effective. Furthermore, insulin led to an increase in synthesis by normal diaphragm in normal serum at a glucose concentration of 0·8%. The effect of insulin under these conditions was, however, irregular; only in 5 out of 10 experiments could an increase in synthesis be induced. Incubation in the serum of alloxan-diabetic insulin-treated rats, or in a medium containing equal volumes of diabetic and of normal serum concentrate, resulted in an increase in synthesis in the diabetic diaphragm. Addition of insulin in the usual or even higher dose had no effect on the synthesis in the diaphragm of alloxan-diabetic acidotic rats (Table 6).

the respiration of normal diaphragm was higher than that of diabetic diaphragm, whereas in diabetic serum no difference could be observed. In experiments with serum only the oxygen uptake was measured, since the direct Warburg method proved unsuitable for the estimation of the CO_2 output (Table 7).

(6) *Glycogen synthesis in liver slices of alloxan-diabetic rats*

In this experiment we were handicapped by the fact that the livers of diabetic animals after fasting still contained appreciable quantities of glycogen (Tuerkischer & Wertheimer, 1946), and a glycogen content of 0·2–0·3% is considered to be the upper

* The respiration experiments were carried out by Mrs I. Salomonowicz-Parnes.

Table 7. *Respiration of diaphragm of alloxan-diabetic and normal rats*

	Alloxan-diabetic rats				Normal rats			
		O_2	CO_2			O_2	CO_2	
Medium	No. of exp.	(μl./mg. dry wt./hr.)		R.Q.	No. of exp.	(μl./mg. dry wt./hr.)		R.Q.
Phosphate-Ringer	10	$-5{\cdot}98 \pm 0{\cdot}29$	$4{\cdot}83 \pm 0{\cdot}27$	$0{\cdot}81 \pm 0{\cdot}010$	17	$-3{\cdot}68 \pm 0{\cdot}80$	$2{\cdot}68 \pm 0{\cdot}61$	$0{\cdot}73 \pm 0{\cdot}016$
Phosphate-Ringer +0·1% glucose	16	$-6{\cdot}71 \pm 0{\cdot}33$	$5{\cdot}90 \pm 0{\cdot}25$	$0{\cdot}88 \pm 0{\cdot}013$	16	$-4{\cdot}47 \pm 0{\cdot}67$	$3{\cdot}90 \pm 0{\cdot}68$	$0{\cdot}87 \pm 0{\cdot}024$
Normal serum, glucose average 0·12%	9	$-5{\cdot}41 \pm 0{\cdot}22$	—	—	9	$-6{\cdot}85 \pm 0{\cdot}29$	—	—
Diabetic serum, glucose average 0·5%	5	$-5{\cdot}42 \pm 0{\cdot}32$	—	—	9	$-5{\cdot}40 \pm 0{\cdot}22$	—	—

limit for the demonstration of synthesis. In acidotic alloxan-diabetic rats, where no glycogen is to be found in the liver, synthesis of glycogen from added glucose was markedly diminished. In these animals the liver is already severely damaged, as shown by the high fat content. Rats suffering from a mild diabetes with normal fasting blood sugar, on the other hand, did not differ from normal rats in regard to the synthesis of glycogen.

DISCUSSION

The effect of potassium on the synthesis of glycogen and glycogenolysis

Potassium in concentrations which are present in extracellular fluid inhibits the synthesis of glycogen from added glucose in rat diaphragm, and complete removal of potassium produces optimal conditions for synthesis. In the liver, on the other hand, synthesis of glycogen will take place only in a medium containing appreciable amounts of potassium (Hastings & Buchanan, 1942), and with this organ the 'extracellular' medium favours glycogenolysis. The same medium thus has an opposite effect on identical processes in liver and muscle. Since even physiological quantities of potassium affect synthesis and favour glycogenolysis in the diaphragm, one must assume that potassium is of physiological importance in the regulation of synthesis and breakdown of glycogen. It is known that part of the body potassium occurs in a free condition, and is able to move about in response to changes in membrane equilibrium. For instance a decrease in the potassium content of working muscle has been adequately demonstrated by Fenn (1940) and Hahn & Hevesy (1941). Fenn assumes that, on its way from muscle to liver, potassium migrates together with lactic acid, and on its way from liver to muscle with glucose. Boyer, Lardy & Phillips (1942, 1943) explain the effect of potassium as being due to an acceleration of the transfer of phosphate from 2-phosphopyruvate to the adenylic acid system. Substitution of potassium by the ammonium ion in equimolar concentration had the same effect. In the experiments of Boyer *et al.* calcium counteracted the effect of potassium. In our experiments, on the other hand, no antagonism between potassium and calcium was found. This might be because the above authors used minced or homogenized muscle, while in our experiments tissue was used which had suffered minimal damage and calcium might be unable to enter the intact cell. However, the role of potassium in the phosphorylating process of the adenylic acid system does not provide an explanation of the opposite effect of this ion on identical processes in liver and muscle tissue. Serum, on the other hand, contains a factor which counteracts the effect of potassium. Owing to the presence of this factor, serum is an optimal medium for glycogen synthesis in the diaphragm. The cause of this effect is still to be explained.

The effect of adrenaline on the synthesis and breakdown of glycogen

The complete inhibition of synthesis and the increase of glycogenolysis in rat diaphragm on addition of small doses of adrenaline is in accord with the findings of Hegnauer & Cori (1934), Cori & Cori (1936) with muscle and Nachmansohn (1937) with muscle *brei*. These authors found that adrenaline increased the formation of hexosemonophosphate and of lactic acid in frog muscle under aerobic as well as under anaerobic conditions. However, whereas in their experiments these metabolic changes were not inhibited by ergotamine, in our experiments the inhibition of the synthesis of glycogen by adrenaline was completely abolished by ergotamine; this may be considered as evidence that in this case adrenaline acts by way of the sympathetic nervous supply still present in the preparation.

Synthesis of glycogen in alloxan-diabetic rats

The *in vitro* synthesis of glycogen in diaphragm from alloxan-diabetic rats showed the following characteristics: the difference between normal and

diabetic diaphragm became apparent only when both serum and diaphragm from alloxan-diabetic rats were used. Under such conditions there was a marked decrease in synthesis with diabetic animals, whilst addition of insulin to diabetic serum greatly increased the synthesis. A similar increase in synthesis could be produced by incubation with serum of insulin-treated alloxan-diabetic rats, with serum of normal rats, or by addition of a normal serum concentrate to the diabetic serum. Incubation with diabetic serum, on the other hand, diminished synthesis of glycogen in the normal diaphragm.

This effect of insulin on diabetic diaphragm incubated with diabetic serum failed to appear when the diaphragms of severely diabetic rats with acidosis were used. In these experiments the known resistance of comatose alloxan-diabetic rats to insulin (Kaplan, Franks & Friedgood, 1945) was corroborated *in vitro*. In buffered salt solutions, however, no significant difference existed between the synthesis of glycogen in normal and diabetic diaphragms. The effect of insulin on the synthesis in diabetic diaphragm with these media was even smaller than that with normal diaphragm. The insulin effect, always present in buffered salt solution, could not be produced in the normal diaphragm incubated with normal serum. In the diabetic diaphragm this effect was definitely present.

An explanation of most of these findings can be given as follows: in normal serum a factor favouring synthesis of muscle glycogen, possibly insulin, is present, and synthesis cannot be improved by addition of insulin. At high glucose concentrations the amounts of this factor present may not be sufficient, and under these conditions addition of insulin may produce an increase in the synthesis of glycogen. In buffered salt media, therefore, an insulin effect could always be produced. In diabetic serum the concentration of this factor is either diminished, or the factor is absent and synthesis of glycogen is therefore decreased. However, this synthesis can be increased by addition of insulin, or by incubation with serum of insulin-treated alloxan-diabetic rats, or by addition of a normal serum concentrate. We can offer no explanation for the diminished effect of insulin in the diabetic diaphragm in buffered salt media.

So far mention has been made only of a factor present in normal and absent from diabetic serum which favours the synthesis of glycogen. But there still exists the possibility that in normal serum two substances may be present, one favouring and the other inhibiting synthesis. Such an assumption would explain the fact that syntheses by the normal diaphragm in the optimal buffered saline and in serum are of the same order of magnitude. Further experiments are required to investigate the validity of this assumption. On the other hand, it is probable that alloxan diabetes may also produce changes in muscle, even if such changes are not apparent in buffered salt media. That a disturbance must exist in the diabetic diaphragm itself is proved by the absence of the insulin effect in the diaphragm of alloxan-diabetic comatose rats incubated with serum from alloxan-diabetic non-comatose rats, and by the effectiveness of insulin on the diabetic diaphragm incubated with normal pooled serum.

In any case these results support the view that important factors influencing the normal carbohydrate metabolism of tissue slices are present in serum. Experiments on synthesis of glycogen as well as on respiration demonstrate that the use of serum in place of buffered salt media may lead to entirely different results (Shipley, 1944). Disturbances in metabolism may become detectable *in vitro* only when the experiments are carried out in the abnormal pathological environment and not in indifferent salt solutions.

SUMMARY

1. Glycogen synthesis and glycogenolysis by rat diaphragm and liver have been studied *in vitro*.

2. Potassium, even in physiological concentrations, inhibited synthesis of glycogen from added glucose by rat diaphragm. At higher concentrations of potassium glycogenolysis took place instead of synthesis. For glycogen synthesis in rat-liver slices, however, it is essential to have potassium present in the medium.

3. Adrenaline completely inhibited synthesis of glycogen in diaphragm and favoured glycogenolysis, whilst ergotamine abolished the effect of adrenaline.

4. Insulin favoured synthesis of glycogen from added glucose in all buffered salt media. With serum, however, no additional synthesis of glycogen could be induced by addition of insulin.

5. Incubation with 'diabetic' serum decreased the synthesis of glycogen in the 'diabetic' as well as in the 'normal' diaphragm. The synthesis in the 'diabetic' diaphragm could be increased by addition of insulin or of a normal serum concentrate to 'diabetic' serum, or by incubation with serum of insulin-treated alloxan-diabetic rats. In normal pooled serum a distinct increase in synthesis was obtained in 'diabetic' but not in 'normal' diaphragm by addition of insulin.

6. No differences, however, between the synthesis of glycogen in diaphragms of alloxan-diabetic and normal rats were observed in the different salt media. The effect of insulin in the 'diabetic' diaphragm in buffered salt media was even smaller than that observed in 'normal' diaphragm.

7. Glycogenolysis in diaphragm was favoured by potassium but unaffected by insulin. The order of magnitude of glycogenolysis was the same in 'normal' and 'diabetic' diaphragm.

8. Metabolic changes of tissues *in vitro* may become manifest only in a correspondingly changed medium and may be undetectable in an indifferent salt medium.

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REFERENCES

Bendall, J. R. & Lehmann, H. (1941). *Nature, Lond.*, **148**, 538.

Boyer, P. D., Lardy, H. A. & Phillips, P. H. (1942). *J. biol. Chem.* **146**, 673.

Boyer, P. D., Lardy, H. A. & Phillips, P. H. (1943). *J. biol. Chem.* **149**, 529.

Cori, C. F. & Cori, G. T. (1936). *J. biol. Chem.* **116**, 119.

Cross, M. C. A. & Holmes, E. (1937). *Brit. J. exp. Path.* **18**, 370.

Dixon, M. (1943). *Manometric Methods.* New York: Macmillan.

Fenn, W. O. (1940). *Physiol. Rev.* **20**, 377.

Gemmill, C. L. (1940). *Johns Hopk. Hosp. Bull.* **66**, 232.

Gemmill, C. L. (1941). *Johns Hopk. Hosp. Bull.* **68**, 329.

Gemmill, C. L. & Hamann, L. Jr. (1941). *Johns Hopk. Hosp. Bull.* **68**, 50.

Gey, G. O. & Gey, M. K. (1936). *Amer. J. Cancer*, **27**, 45.

Good, C. A., Kramer, H. & Somogyi, M. (1933). *J. biol. Chem.* **100**, 485.

Hahn, L. & Hevesy, G. (1941). *Acta physiol. Scand.* **2**, 51.

Hastings, A. B. & Buchanan, J. M. (1942). *Proc. nat. Acad. Sci., Wash.*, **28**, 478.

Hechter, O., Levine, R. & Soskin, S. (1941). *Proc. Soc. exp. Biol., N.Y.*, **46**, 390.

Hegnauer, A. H. & Cori, G. T. (1934). *J. biol. Chem.* **105**, 691.

Kanner, O. (1946). *Science*, **104**, 253.

Kaplan, N., Franks, M. & Friedgood, C. E. (1945). *Science*, **102**, 447.

Nachmansohn, M. D. (1937). *Bull. Soc. Chim. biol., Paris*, **19**, 453.

Ostern, P., Herbert, J. & Holmes, E. G. (1939). *Biochem. J.* **33**, 1858.

Saxton, S. A. & Miller, M. L. (1944). *Arch. Path. Lab. Med.* **37**, 34.

Shipley, R. A. (1944). *Amer. J. Physiol.* **141**, 662.

Somogyi, M. (1937). *J. biol. Chem.* **117**, 771.

Stadie, W. C. (1944). *Yale J. Biol. Med.* **16**, 539.

Tuerkischer, E. & Wertheimer, E. (1946). *Nature, Lond.*, **158**, 201.

Van Dyke, H. B. & Hastings, A. B. (1928). *Amer. J. Physiol.* **83**, 563.

Hydrochloric Acid Production by Isolated Gastric Mucosa*

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With an Appendix by R. E. Davies and F. J. W. Roughton

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The experiments described in this paper were undertaken to throw light on the mechanism of hydrochloric acid production in the stomach. Isolated mucosa was used because it is easier to control the experimental conditions in excised tissue than in the intact organism.

The oxyntic cells of rats secrete continuously (Friedman, 1943), hence the starting materials for the production of HCl must come eventually from the blood and must be neutral. Several mechanisms which produce hydrogen ions have been envisaged and can be divided roughly into two types. (1) Those producing the acid together with approximately neutral compounds. (2) Those producing the acid together with basic compounds (Fig. 1). Many manometric experiments have been carried out in Warburg cups with sheets of sheep, toad and frog gastric mucosa to test the possibility that mechanisms of type 1 are involved. In no case was evidence obtained that HCl was being formed, and liberating CO_2 from the HCO_3^- of the medium, although the rate of respiration of the tissues was normal and was increased by histamine. Since several glands can function *in vitro* (Delrue, 1930; Deutsch & Raper, 1936) it seemed unlikely that the failure to detect acid was due to the inability of the

* A part of the present work was communicated to the Biochemical Society (Davies, 1946).

mucosa to secrete it. These negative results suggest that mechanisms of type 1 are not operative, as would be expected if the old hypothesis of Bence Jones (1845) that 'the formation of acid sets free alkali' were true, assuming that the acid and alkali are formed in equal amount as in the type 2 mechanisms.

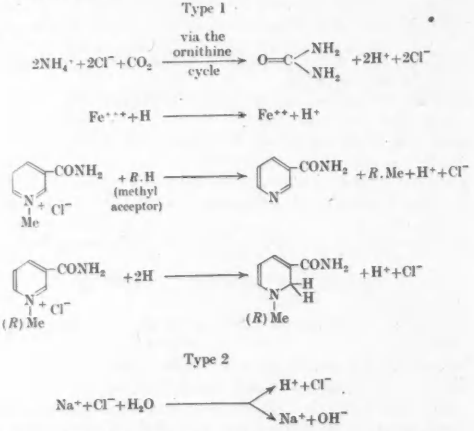

Fig. 1. Some biochemical reactions producing hydrogen ions. Type 1. Those producing the acid and approximately neutral compounds. Type 2. Those producing the acid and basic compounds. Type 1 mechanisms which are also reversed within the cell become type 2 mechanisms.

Experiments were devised to combine collection and estimation of the secretions with manometric measurements of the respiratory activity of the tissue; they show that when gastric mucosa secretes acid, an equivalent amount of alkali is also produced in the cell, which normally is at once neutralized by CO_2 and secreted into the surrounding medium.

METHODS

Saline media. The physiological salt solution (bicarbonate saline) of Krebs & Henseleit (1932), gassed with 5% (v/v) CO_2 + 95% (v/v) O_2, or 5% CO_2 + 95% N_2, was used during measurements of the balance of the O_2 uptake and the CO_2 output, or of the anaerobic glycolysis of sheep gastric mucosa, and the phosphate saline of Krebs (1933) during experiments in 100% O_2. The glucose concentration was 0·2 or 0·4%. In experiments with amphibian tissue these two solutions were used, after the addition of 0·29 vol. of water, and contained 0·2% (w/v) glucose. Another solution was used in the preparation of the mucosa and during some experiments in 100% O_2. It was a basal saline similar to amphibian bicarbonate saline, but contained 0·004 M-HCO_3^-.

Preparation of the gastric mucosa. Sheep, frog and toad gastric mucosae were used. The abomasum was removed from the sheep within 5 min. of electric stunning, and carried rapidly to the laboratory in ice-cold 0·16 M-NaCl solution. After thorough washing a mucosal flap was dissected away, opened out and freed from submucosa. Pieces of tissue with a dry weight of 20–40 mg. were cut off and used at once. They were about 0·3–0·6 mm. thick, and did not curl up tightly.

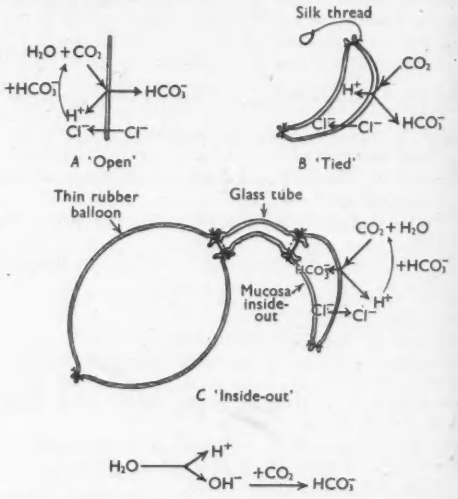

Fig. 2. Diagrammatic representation of the overall reactions in a type 2 mechanism in *A* 'open', *B* 'tied' and *C* 'inside-out' frog gastric mucosa in saline media containing bicarbonate ions. In *A* and *C* the CO_2 liberated from the medium by the H^+ is equivalent to that needed to neutralize the OH^-, so no supplies of CO_2 are needed. In *C* the decrease of the HCO_3^- content in the external fluid is equivalent to the increase in the internal fluid. In *B* the acid secretion is collected inside the mucosal bag and supplies of CO_2 are needed to neutralize the OH^-.

Frogs (*Rana temporaria temporaria* L.) and toads (*Bufo bufo bufo* (L.)) were captured locally and housed in a concrete froggery at 15–17° after the web had been tattooed with Indian ink for identification. An excess of cockroaches or worms was provided for food. The amphibia were pithed as required, and the stomach dissected out after cutting the oesophagus and duodenum. It was emptied by gentle squeezing and washed by passing 5 ml. of basal saline through it from a pipette. In none of more than 400 cases has any residual acid been found in the stomach after this procedure. The following technique was evolved for isolating the intact gastric mucosa. The stomach, with the greater curvature upwards, was gently pressed with the left thumb and index finger to tighten the muscle coats. A small longitudinal incision was made with a razor, and continued to both ends with fine scissors without damaging the mucosa. Starting from the pyloric end, the intact tube of mucosa was separated from the muscles with fine forceps and pulled free. It was then blotted and rapidly weighed on a torsion balance; with care the weights were reproducible to 1 mg. During the dissection the tissue was frequently damped with basal saline.

Amphibian tissue was used in three different ways: 'open', 'tied' and 'inside-out'. For experiments with open tissue the mucosa was cut along the greater and lesser curvatures and used directly. Tied tissue was used when collection of the secretion was required; the pyloric end of the mucosa was closed with fine silk thread, and the lumen completely emptied by gentle stroking with curved forceps. The cardiac end was next tied, and a loop made in the silk with which to hang the preparation on the hook of the torsion balance. Excess silk was cut off and the tissue blotted and reweighed. After clipping off the spare tissue past the knots, the unit was reweighed and was ready for use. The time from pithing the amphibian to placing the preparation in the cup was usually 9–12 min.

The balance of O_2 uptake and CO_2 output of a tied inside-out tube of acid-secreting mucosa should be similar to that of open sheets of acid secreting tissue, if as much alkali as acid is formed (Fig. 2). In this case, however, it is possible to prove that secretion occurs, by estimating the changes in the HCO_3^- content of the internal and external media.

The lumen of an inside-out gastric mucosa is not big enough to hold sufficient medium to supply the tissue, so a source of nutrient was attached to it. Tiny glass U-tubes were made, and a 4 ml. balloon of very thin rubber tied to one arm. A clean intact mucosa was then turned inside-out by passing fine forceps through the cardiac sphincter, seizing the pyloric end and sliding the tissue along the instrument. After tying the pyloric end, the cardiac end was attached to the other arm of the U-tube. The nutrient solution was injected into the balloon with a hypodermic syringe, and the air inside the preparation completely sucked out. After tying off the puncture hole, to prevent leakage, the preparation was weighed and was then ready for use (Fig. 2).

Manometric procedure. The O_2 uptake, the resultant of O_2 uptake and CO_2 output, and the anaerobic glycolysis were measured with Warburg manometers in the usual way at 40·0° with mammalian, and 25·0° with amphibian tissue. Conical cups (20–30 ml. capacity), with a centre well, were used for most of the experiments. Histamine and other agents were added from the side arms, or from Keilin tubes (Keilin, 1929). The values of the Q_{O_2}, Q_{CO_2} and Q_{HCl} are in μl./mg. dry wt./hr.

Final treatment of tissues. At the end of the incubation, open tissue was washed in distilled water and dried overnight at 110°. Tied bags of mucosa, now distended by secretions, were uniformly blotted, the small blobs of mucus formed at the rosette ends removed, and the residue hung on the torsion balance by the silk loop and weighed. The contents were drained into a small sample tube, after cutting open the swollen bag of mucosa, which was reweighed when empty. The silk was removed and the tissue washed and dried as before. From the various weights, both the amount of secretion and the total content of the isolated mucosa can be estimated.

When the tissue had been used inside-out, care was taken to drain off as much as possible of the external fluid, whose volume was measured after transference to a 5 ml. graduated tube. The balloon, glass tube and mucosa were then blotted, weighed, and the internal fluid drained as completely as possible into a 5 ml. graduated tube. The volume of liquid transported by the mucosa could thus be calculated. The tissue was cut off, washed and dried as before.

Estimation of the amount of acid secreted. A sample (10–100 mg.) of the gastric contents was weighed into the side arm of a Warburg cup, and, after equilibration at 25° with 5% CO_2 + 95% O_2, was tipped into 4 ml. of a solution containing 0·02 M-$NaHCO_3$ and 0·1 M-NaCl. This solution is isotonic with frog serum and secretion, so no gas exchanges due to differences in vapour pressure occur on mixing. From the output of CO_2 the acidity of the gastric contents can be calculated to an end point of pH 7·4. This pH was chosen because the secretion was ultimately formed from the medium, which had a pH of 7·4.

Bicarbonate estimation. After removing the tissue, a sample of the medium was placed in the main compartment of a Warburg cup and equilibrated at 25° with 5% CO_2 + 95% O_2. 0·1 ml. N-HCl per ml. of medium was then added from the side arm. The output of CO_2 was compared with that from a sample of the original medium, to determine the change in bicarbonate concentration. Since CO_2 is less soluble in acid than in neutral solutions (Dixon, 1943), this technique does not give directly the absolute quantity of bicarbonate in either solution, but gives a sufficiently accurate value of the small difference between the two solutions.

Lactic acid was determined by the method of Friedemann & Graeser (1933).

RESULTS

A. *The metabolism of sheets of gastric mucosa*

Sheep gastric mucosa. It is necessary to know whether the tissue is sufficiently thin to allow adequate amounts of O_2 to diffuse into it to maintain its metabolic activity. The thickness of samples of sheep abomasal mucosa, determined from the weight of a known area, was from 0·3 to 0·6 mm., average 0·5 mm., assuming a density of 1. The ratio dry weight/wet weight was 0·2. The formula of Warburg (1923), which is of doubtful validity when applied to a tissue such as gastric mucosa, which contains cells of differing metabolic activities, gives the upper limiting value of the $|Q_{O_2}|$ as 16 at 38° at which the O_2 pressure at the centre of the tissue is just zero. The Q_{O_2} was measured in 56 out of 127 experiments, and was between $-2·9$ and $-7·0$, average $-4·7$, standard deviation 0·81. Sheep mucosa thus appeared to be a satisfactory tissue for the study of HCl secretion.

When the tissue was incubated aerobically in Warburg cups, the Q_{O_2} and Q_{CO_2} had the usual magnitudes in bicarbonate or phosphate salines or serum, and there was a slight excess CO_2 evolution which corresponded with the lactate formed in the medium. No excess CO_2 evolution due to HCl reacting with HCO_3^- in the media was detected, even after the addition of histamine to concentrations from 5×10^{-8} to 5×10^{-4} M, though in the middle of this range the Q_{O_2} was usually increased by 5–20%. Variations in the tissue/medium ratio had no effect, nor did the addition of extracts of gastric mucosa, so failure to detect acid secretion did not appear to be caused by a loss of diffusible coenzymes.

Various compounds, which might be expected to take part in mechanisms of type 1, were added to discover their effect on the Q_{O_2}. Acetyl choline and eserine, at concentrations from 5×10^{-7} to 5×10^{-4}M, did not alter the Q_{CO_2}, whilst 0·005 or 0·0025M-NH_4^+ had no effect and was not utilized, either alone or together with 0·005 or 0·0025M-ornithine, in the presence or absence of histamine or acetyl choline and eserine. 0·0025M-nicotinamide methochloride, either alone or together with 0·0025M-glycocyamine, was without effect on the Q_{O_2} or Q_{CO_2} of sheep abomasal mucosa.

Frog gastric mucosa. Sheets of frog gastric mucosa also failed to show evidence of acid production, but, after the addition of histamine to a concentration of 5×10^{-5}M, the rate of O_2 uptake was usually increased, often by 10–20%, but sometimes by more than 100% (Fig. 3). No change was observed when

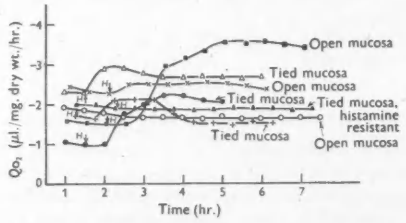

Fig. 3. Typical response curves of seven frog gastric mucosae. Open sheets and tied tubes of mucosa incubated in nutrient phosphate saline gassed with O_2 at 25·0°. Histamine added in six cases to a concentration of 5×10^{-5}M at the arrows marked *H*.

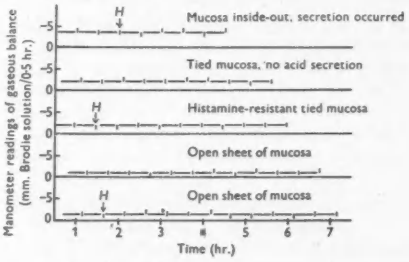
Mucosa inside-out, secretion occurred
Tied mucosa, no acid secretion
Histamine-resistant tied mucosa
Open sheet of mucosa
Open sheet of mucosa
H
Manometer readings of gaseous balance (mm. Brodie solution/0·5 hr.)
Time (hr.)

Fig. 4. The balance of O_2 uptake and CO_2 output in five frog gastric mucosae. Preparations incubated at 25·0° in nutrient bicarbonate saline gassed with 5% CO_2 + 95% O_2. Histamine added in three cases to a concentration of 5×10^{-5}M at the arrows marked *H*. The vertical lines represent the readings and their probable error.

the balance of O_2 uptake and CO_2 output was measured (Fig. 4). This means that if HCl was produced an equivalent amount of alkali was also formed.

When a complete gastric mucosa was cut across to give samples of tissue from the cardiac, corpus and pyloric regions, which were then incubated, in all of three cases the pyloric mucosa had a low Q_{O_2} and failed to respond to histamine, whilst the other two were more active and responded in accordance with their content of oxyntic cells (Table 1). In all of four

Table 1. *Effect of* 5×10^{-5}M-*histamine on* O_2 *uptake of various regions of frog gastric mucosa*

(A. Mucosa cut across into three sheets of approx. equal area, consisting largely of cardia, corpus and pyloric antrum of dry weight 20·1, 16·1 and 9·4 mg. respectively. B. Mucosa cut lengthways into three strips of approx. equal area and dry weight. Incubated at 25·0° in 4 ml. phosphate saline containing 0·2% glucose. Histamine added from side arm after 2 hr. Duration of response at least 5 hr.)

Source of mucosa	Av. Q_{O_2} before histamine (µl./mg. dry wt./hr.)	Max. Q_{O_2} after histamine (µl./mg. dry wt./hr.)
A		
Cardia	−1·4	−2·7
Corpus	−1·5	−3·5
Pyloric antrum	−1·2	−1·3
B		
Greater curvature	−1·0	−2·6
Posterior region	−0·9	−2·5
Anterior region	−0·9	−2·4

cases strips from a single mucosa, obtained by cutting the tissue from pylorus to oesophagus, had similar Q_{O_2} and Q_{CO_2} values to within about 10%. This fact was utilized to compare the O_2 uptake of a strip in phosphate saline with the uptake calculated from the gaseous exchanges of two similar strips from the same mucosa in bicarbonate saline, using the indirect method of Warburg (Dixon, 1943). The use of this indirect method of measuring the Q_{O_2} presupposes that the ratio of tissue to medium has no effect on the O_2 uptake. This was checked by using three strips, cut lengthways from both of two mucosae, in phosphate saline. The tissue/medium ratio was varied by a factor of 7·5, and in both series the basal and histamine-stimulated rates agreed within the normal variation.

After a trial of many temperatures between 5° and 40°, 25° was chosen for experiments with frog gastric mucosa, because it is high enough to allow active metabolism, yet safely below 30°, above which temperature the tissue suffers heat damage.

B. *The production of* HCl *by tied intact tubes of frog gastric mucosa*

The equivalence of the acid and alkali produced. When an intact tube of frog gastric mucosa was tied with silk at both ends and placed in a Warburg cup in bicarbonate saline gassed with 5% CO_2 + 95% O_2,

the change in the gas pressure was similar to that with open sheets of tissue. The small negative pressure change was the resultant of O_2 uptake and CO_2 output, and was usually of the order of 1–2 mm. Brodie solution/0·5 hr. The addition of histamine to a concentration of 5×10^{-5}M was usually followed by a large increased uptake of gas (up to 55 mm. Brodie solution/0·5 hr.) (Fig. 5), and the tube of mucosa became distended with secretion containing mucus and HCl. Histamine-resistant mucosae secreted mucus only (Fig. 4, Table 4), and did not have an increased uptake of gas.

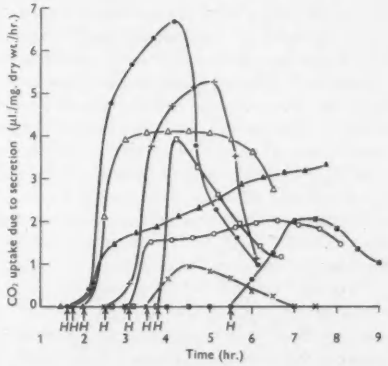

Fig. 5. Typical histamine response curves of eight tied tubes of frog gastric mucosa. Preparations incubated at 25·0° in nutrient bicarbonate saline gassed with 5% CO_2 + 95% O_2. Histamine added in all cases to a concentration of 5×10^{-5}M at the arrows marked *H*.

The following experiments show that this increased gas uptake was due to CO_2 absorption. The experiments on sheets of mucosa showed that the balance of the gas exchanges was not significantly altered by the addition of histamine, and that the loss of HCO_3^- in the medium was accounted for by the amount of lactic acid produced (Table 2). In experiments on acid-secreting closed tubes of mucosa, bicarbonate estimations showed a large increase of this ion in the medium. This increase does not represent the total amount of HCO_3^- formed, because some was decomposed by the lactic acid formed by aerobic glycolysis. The amount of lactic acid in the medium was determined, and a corrected value of the increase of HCO_3^- thus obtained. This value gave good agreement with the extra uptake of gas concomitant with acid secretion, measured in mm. Brodie solution and multiplied by the manometer constant k_{CO_2} for the experimental conditions. This proves that the gas absorbed was CO_2.

The quantity of gas absorbed was also equivalent to the amount of HCl found inside the bag of mucosa (Table 2), and this proves that the mucosa produces

Table 2. *Relation between change in gaseous balance, HCl, HCO_3^- and lactate production in typical frog gastric mucosae*

(Open or tied mucosae were placed in 4 ml. nutrient bicarbonate saline and the gaseous balance in 5% CO_2 + 95% O_2 followed at 25·0°. After the experiments the weight of secretions and the amount of acid in the mucosal bag and of HCO_3^- and lactate in the medium were determined.)

	Dry wt. of tissue (mg.)	Wet wt. of secretion (mg.)	HCl produced (µl.)	CO_2 uptake due to secretion (from change in gaseous balance) (µl.)	ΔHCO_3^- in medium (µl.)	Lactate formed (µl.)	ΔHCO_3^- + lactate (µl.)	HCl $-(\Delta HCO_3^-$ + lactate) (µl.)	Duration of experiment (hr.)	Time of addition of histamine (hr.)
A. Histamine to 5×10^{-5}M added during experiment										
Tied	12·6	86 ± 1	+ 183 ± 2	+ 180 ± 2	+ 154 ± 6	+ 38 ± 4	+ 192 ± 10	- 9 ± 12	5½	1½
Tied	13·8	32 ± 1	+ 41 ± 2	+ 40 ± 2	+ 13 ± 6	+ 23 ± 4	+ 36 ± 10	+ 5 ± 12	4	1
Open	15·5	—	—	0 ± 1	- 24 ± 6	+ 25 ± 4	+ 1 ± 10	—	6	1½
B. No histamine added										
Tied	20·0	11	0 ± 1	0 ± 1	- 33 ± 6	+ 28 ± 4	- 5 ± 10	+ 5 ± 11	6½	—
Open	23·9	—	—	0 ± 1	- 40 ± 6	+ 33 ± 4	- 7 ± 10	—	5	—

alkali, which is neutralized by CO_2 at the same rate as it secretes acid. The changes in the rate of gaseous uptake can therefore be used to follow the changes in he rate of HCl secretion during the experiment. It should be pointed out that, if the ratio volume of fluid (V_F)/volume of gas (V_G) is made large, then the change in the metabolic rate of the tissue after stimulation with histamine can cause an appreciable change in the now large resultant balance of the gas exchanges, which does not occur if the ratio V_F/V_G is small, as in the experiments described (cf. Warburg, 1926).

The relationship between the Q_{O_2} and the Q_{HCl}. In order to correlate HCl production with O_2 uptake, experiments were carried out in phosphate saline gassed with O_2, with alkali in the centre well of the Warburg cup. The increase in the Q_{O_2} following the addition of histamine (Fig. 3) was associated with acid production, and was less for small than for high rates of secretion (Table 4). In solutions such as phosphate saline and blood, which contain buffers other than the H_2CO_3:HCO_3^- system, retention occurs so that, although the mucosa still produces as much alkali as acid, the HCO_3^- formed reacts with these buffers when it is secreted into the medium. Experiments using frog serum showed that the metabolic activities of frog gastric mucosa were indistinguishable in this medium from those in nutrient saline solutions.

From a knowledge of the magnitudes of the Q_{O_2} and Q_{HCl} during secretion it is possible to assess some theories of the elaboration of HCl by gastric mucosa. A difficulty is that when the variations in O_2 uptake in a Warburg cup are measured, only the average Q_{HCl} can be estimated from the total amount of HCl produced by the mucosa, and this is less than the maximum value. If the variations in the gaseous balance are followed, the Q_{O_2} of the tissue is not known, and the regional differences in the tissue preclude the use of the material clipped off outside the knots to find the Q_{O_2}.

The maximum $|Q_{O_2}|$ of 92 stimulated mucosae was from 1·1 to 4·3, average 2·6, standard deviation 0·56. The maximum $|Q_{O_2}|$ of 28 sheets of stimulated mucosal tissue was from 1·0 to 3·8, average 2·5, standard deviation 0·57. This shows that tied mucosae were effectively oxygenated by diffusion from one side of the tissue only. The largest frog so far used weighed 64 g. and its mucosa was 0·29 mm. thick. With $|Q_{O_2}|$ values < 5 this thickness is well within the safety limits given by Warburg's (1923) formula, even if the tube of mucosa is tied. The maximum Q_{HCl} observed in 130 experiments (98 with males) was 8·1 and in 20 cases (16 males) the maximum Q_{HCl} rose to more than 4. No sex difference is thus apparent. Secretion could last for only 1 hr. or for at least as long as 8 hr. but usually stopped 4–6 hr. after the addition of histamine (Fig. 5).

All the mucosae with high average rates of secretion were in bicarbonate saline, and it is therefore important to know the Q_{O_2} of secreting tied mucosae in this medium. The ordinary way of calculating the results, using the second method of Dickens & Šimer (Dixon, 1943), gives an apparent negative glycolysis with secreting tied mucosae. Since with open tissue the amount of glycolysis and the lactic acid found in the medium are in agreement, the true glycolysis in tied tissue can be found by estimating the lactic acid formed, and thus the Q_{alkali} of the mucosa can be calculated. As previous experiments have shown, this is equal to the Q_{HCl}. The disadvantage of this method is that only the overall rates of O_2 uptake, respiratory CO_2 output and glycolysis can be measured, though an estimate of the variations in the Q_{HCl} can be obtained from the changes in the gaseous uptake after histamine stimulation. The mean average Q_{O_2} in 20 experiments in bicarbonate saline was $-2·6$ and in four cases the average Q_{HCl} over the whole period of the experiment was greater than the average $|Q_{O_2}|$. From the normal shapes of the response curves (Figs. 3, 5) it seems probable that in these experiments the maximum Q_{HCl} was much higher than the maximum $|Q_{O_2}|$. In any case, only that part of the tissue between the silk loops produced the acid found inside the mucosal bag, whilst the whole unit respired. No account has been taken of this in the calculations, so the evidence is overwhelming that the Q_{HCl} can be higher than the $|Q_{O_2}|$ in isolated frog gastric mucosa provided with external supplies of CO_2. Several toads have been used, and mucosae from this amphibian behaved in every way similarly to frog mucosae.

The response to histamine. Histamine was more effective at 5×10^{-6}M than in more dilute solutions such as 10^{-6} or 10^{-7}M. This concentration of 5×10^{-5}M is relatively high, but, since the gastric mucosa removes histamine and is relatively thick compared with the distance from the blood capillaries to the oxyntic cells, diffusion will be slow and the actual concentration of histamine in the central layers of the tissue, which contain the oxyntic cells, will be much smaller than 5×10^{-5}M, so the necessity for this high concentration in the medium for optimal results may not be unexpected.

Some mucosae failed to secrete HCl even after the addition of histamine to a concentration of 5×10^{-3}M. The fraction of histamine-resistant mucosae was lower in autumn 1945 and spring 1946 than during the winter; spontaneous secretion appears to be largely a summer phenomenon and did not occur during the investigations described in this paper.

Experiments with 'inside-out' mucosa. Tubes of mucosa turned inside-out, and attached to a supply of nutrient saline in small balloons (Fig. 2), were

placed in nutrient saline in a Warburg cup, and the gaseous balance followed in four cases whilst the O_2 uptake was observed in another four experiments. The manometric readings obtained with the inside-out tissue were the same as those obtained with open sheets, both before and after the addition of histamine. This is to be expected only if no secretion occurred, or if the acid secreted into the external fluid liberated a quantity of CO_2 equivalent to that diffusing through the mucosa to neutralize the alkali formed concomitantly with the acid (Fig. 2). Proof that histamine is effective from the acid-secreting side of the mucosa, and that secretion did take place, was obtained by estimating the change in liquid volume and $[HCO_3^-]$ in the internal and external fluids. These estimations show that the acid and alkali are equivalent, with a possible error of about 10% (Table 3).

Table 3. *Secretion by mucosa turned inside-out*

(Inside-out frog gastric mucosae tied at pyloric end and attached by bent glass tubes to thin balloons containing 2 ml. nutrient bicarbonate saline. The units then placed in 2 ml. nutrient bicarbonate saline and the gaseous balance in 5% CO_2+95% O_2 followed at 25·0° before and after addition of histamine to a concentration of 5×10^{-5} M at 2 hr. The experiments stopped at 4 hr. and estimations of HCO_3^- and lactate made on internal and external solutions. Typical results.)

	Exp. 1	Exp. 2
Dry wt. of tissue (mg.)	20·6	34·3
Fluid transport (ml.)	0·250±0·010	0·200±0·010
ΔHCO_3^- internal (μl.)	+61±2	+36±2
ΔHCO_3^- external (μl.)	−73±2	−58±2
Lactate internal (μl.)	+ 8±4	+15±4
Lactate external (μl.)	+ 2±4	+ 5±4
ΔHCO_3^- internal due to secretory activity (μl.)	+69±6	+51±6
ΔHCO_3^- external due to secretory activity (μl.)	−71±6	−63±6
Change in gaseous balance after histamine (mm. Brodie solution)	0±0·5	0±0·5

The anaerobic metabolism of frog gastric mucosa. Unstimulated or histamine-resistant tied mucosae secreted appreciable amounts of neutral mucus (Table 4). In the absence of O_2 none of four mucosae produced any secretion. The preparations were as flat and empty at the end of the experiment as at the beginning, and the weight was unaltered. The anaerobic glycolysis, average +1·9 μl./mg. dry wt./hr., was quite unaffected by the addition of histamine. Whilst aerobically the tissue could respire and secrete HCl, for some hours at least, in the absence of external supplies of glucose, anaerobically the glycolysis fell off rapidly under these conditions (cf. Lutwak-Mann, 1947).

Table 4. *Amount and concentration of acid in secretion, aerobically and anaerobically*

(Typical results for tied tubes of mucosa placed in 4 ml. of nutrient phosphate saline gassed with O_2, or nutrient bicarbonate saline gassed with 5% CO_2+95% O_2 or 5% CO_2+95% N_2 at 25·0°. Complete anaerobiosis secured with yellow phosphorus in centre well. The tube weighed before and after incubation and after emptying. The differences between the increase in weight of the tube, the 'secretion' and the actual 'contents' are probably accounted for by extrusion of mucus into the lumen of the mucosa.)

Gas	Wet wt. of tissue (mg.)	Dry wt. of tissue (mg.)	Wet wt. of secretion (mg.)	Wet wt. of contents (mg.)	Consistency of contents	HCl produced (μl.)	H^+ in contents (M)	Average Q_{gas}	$\Delta\lvert Q_{O_2}\rvert$ due to histamine	Duration of experiment (hr.)	Time of addition of histamine (hr.)
A. Histamine to 5×10^{-5} M added during experiment											
5% CO_2+95% O_2	109	18·3	52	60	Clear jelly	72	0·055	0·71		7	1½
5% CO_2+95% O_2	120	18·2	156	192	Clear liquid	198	0·046	2·0		6½	1
5% CO_2+95% O_2	148	24·3	198	234	Clear liquid	424	0·081	3·5		6	1
5% CO_2+95% N_2	101	19·7	1	0	—	0	—	0·00		6	2
5% CO_2+95% N_2	81	17·5	0	0	—	0	—	0·00		4	1
O_2	93	13·3	49	59	Whitish jelly	74	0·056	1·2	0·3	5½	1
O_2	119	16·5	81	111	Whitish jelly	151	0·061	1·8	0·6	6	1
B. No histamine added											
O_2	152	36·6	47	55	Clear jelly	0	0·000	0·0	—	6½	—

DISCUSSION

The equivalence of the acid and alkali produced by oxyntic cells. In 1859 Brücke assumed that if a free acid was liberated in the gastric tubules, an alkaline fluid of corresponding strength must at the same time pass into the blood and lymph systems (Brücke, 1859). Peters & Van Slyke (1931) have reviewed the many investigations since then into the acid-base balance of the blood, which have demonstrated an increase in its HCO_3^- content apparently associated with gastric HCl secretion. Hanke, Johannesen & Hanke (1931) found that the amount of alkali liberated by the stomach was roughly proportional to the acid secreted, and claimed that the amounts were therefore approximately equivalent. This claim was repeated by Hanke (1937) without support from published evidence. Delrue (1930) first obtained HCl secretion *in vitro* using two chambers, filled to the same level, separated by a sheet of frog gastric mucosa. Although he showed that the pH of the solution at the sub-mucosal side of the tissue rose during secretion, he apparently did not realize the significance of his results. Gray, Adkison & Zelle (1940) repeated these experiments and obtained results 'suggesting that alkali was being liberated from the muscularis surface'.

The experiments described in this paper show that no secretion of HCl could be detected manometrically when sheets of gastric mucosa were incubated in Warburg cups. When the secretion was kept enclosed within the intact tied gastric mucosa HCl production was readily observed. This result in itself shows, with a possible error of about 1%, that 1 mol. of alkali is produced with each mol. of acid, because the HCl produced by a mucosa of dry weight 40 mg. and a Q_{HCl} 5 μl./mg. dry wt./hr. liberates 200 μl./hr. CO_2, and a change of 1 μl./hr. in the overall gaseous balance can be detected with both inside-out and open tissue. As this change does not occur, 200 ± 1 μl./hr. CO_2 must be absorbed to neutralize the alkali. The experimentally observed equivalence of this acid and alkali, in experiments with the tissue tied and inside-out, supports the conclusion that they are exactly equivalent. This equivalence explains the numerous failures to observe the presence of acid in fresh excised gastric mucosae (Hoerr & Bensley, 1936; Linderstrøm-Lang, Holter & Ohlsen, 1935).

The utilization of external supplies of CO_2 *by oxyntic cells.* As the maximum observed Q_{HCl} is about twice the maximum observed $|Q_{O_2}|$, an actively secreting gastric mucosa may produce acid and alkali at such a rate that a supply of CO_2 in excess of its own respiratory output is needed to neutralize the alkali. There are several reports which support this statement. The neglected work of Edkins & Murray (1924) can now be interpreted as showing that added CO_2 disappears entirely from the lumen of a secreting stomach, and reaches the tissue CO_2 pressure in a non-secreting stomach. McIver, Redfield & Benedict (1926) claimed, by showing that the movement of CO_2 into and out of the stomach is governed by the physical laws of diffusion, that they had refuted this work. As no HCl was secreted during their experiments their claim is unjustified. Kurtz & Clark (1946) recently demonstrated an inverse relationship between the amount of HCl secreted and the amount of CO_2 diffusing into the gastric cavity, whilst Teorell (1933) found negative respiratory quotients using secreting cats' stomachs. These results are all explicable if the actively secreting mucosa utilizes external supplies of CO_2.

The rates of respiration and of acid secretion in mammalian gastric mucosa. The result obtained with frog mucosa that the Q_{HCl} can be greater than the $|Q_{O_2}|$ applies also in mammalian mucosa, as can be shown by calculations based on previously published work. Concomitant measurements of the rates of respiration and secretion of a mammalian stomach have been recorded in at least three papers. Using operative procedures which probably damaged the tissue, as judged by the low activities, Teorell (1933) found that 12 g. cat mucosa used 0·45 ml. O_2/min. and produced up to 0·20 ml. 0·1N-gastric juice/min. The writer has found that the dry weight of cat mucosa is about 20% of the wet weight. Therefore the Q_{O_2} was -11 and the Q_{HCl} was $+11$. Similar values can be obtained from the data of Ni & Lim (1928) and Hanke (1937) who worked on dogs. Since all the acid was produced by the oxyntic cells, but only a fraction of the O_2 was used by them, the Q_{HCl} of these cells must have been much higher than their $|Q_{O_2}|$. However, the rates of acid secretion by these vivi-perfused stomachs were much lower than those for gastric tissue prepared after less operative interference.

In unpublished experiments Mackay & Davies have obtained 51 mg. HCl/15 min. for 2 hr. from a chloralosed cat with an acid-secreting mucosa of dry weight 2·7 g. In this case the Q_{HCl} was 46. Rehm & Enelow (1945) obtained 16 mg. HCl/10 min. from a 21 sq.cm. sheet of dog gastric mucosa. It can be calculated from the figures of Ni & Lim (1928) that their dog's mucosa was 1·3 mm. thick, and it is unlikely that the mucosa used by Rehm & Enelow (1945) was thicker than 1·5 mm. Using this value and a dry weight/wet weight ratio of 0·2 the Q_{HCl} was 93. These values of the Q_{HCl} are far higher than the $|Q_{O_2}|$ of cat and dog mucosa.

Many figures are available for the peak value of secretory activity in man. Glenn (1946) recorded the results of 450 histamine-stimulated fractional gastric analyses. The mode of peak volume (ml./15 min.) $\times$ free acidity (clinical units) was 4000 and the maximum value 16,000. Mrs Bradford and the author

have found the dry weight in g. of 11 human acid-secreting mucosae to be from 8·6 to 15·5, average 12·0, standard deviation 2·0. The mode of the maximum Q_{HCl} was therefore 30. If it is assumed that the subject with the highest rate of secretion had an acid-secreting mucosa of even 16 g., the Q_{HCl} must have been 90. Rosenthal & Lasnitzki (1928) found that the Q_{O_2} of 11 samples of isolated, unstimulated, human gastric mucosa lay between -5 and -20. The average was $-9{\cdot}2$.

Since the R.Q. of any gastric mucosa is unlikely to be much more than 1·0 it is clear that the Q_{HCl} of the oxyntic cells and even of the entire acid-secreting mucosa can rise much higher than both Q_{O_2} and Q_{CO_2} in the frog, cat, dog and man.

Bearing of the relationship between Q_{O_2} and Q_{HCl} on some recent theories of HCl *elaboration.* The findings discussed in the preceding paragraph bear on some recent theories of gastric acid elaboration. Davenport (1943), Bull & Gray (1945) and Conway, Fitzgerald & Walls (1945) have variously suggested that the H^+ ions are produced at the same rate as, or arise from, acids such as pyruvic or carbonic formed during oxidative metabolism within the oxyntic cells. Since 2 mol. of pyruvic acid are formed from 1 mol. of glucose, and 6 mol. of O_2 are required to oxidize the glucose to 6 mol. of CO_2 and 6 mol. of H_2O, in the first case the Q_{HCl} could never be greater than one third of the Q_{O_2} and in the last case the Q_{HCl} could never be greater than the Q_{O_2}. The possibility that the oxyntic cells remove pyruvic acid from the blood, secrete the H^+ ions and metabolize the anions is unlikely, because the cells do not require supplies of this acid in order to produce HCl.

The observed relation between the Q_{HCl} and the Q_{O_2} renders untenable any theory that the H^+ ions are produced by the oxidative degradation of any substrate known to take part in intermediary metabolism. The results of these experiments support the view that the reaction fundamentally concerned in the production of HCl in gastric mucosa is

$$H_2O \rightarrow H^+ + OH^-.$$

The H^+ ions are secreted, and the OH^- ions finally neutralized by CO_2 and passed into the blood or nutrient medium. This is comparable with the membrane hydrolysis theory of Hollander (1943), and explains the CO_2 uptake which occurs during acid secretion.

The function of carbonic anhydrase in oxyntic cells. Davenport (1939) found that oxyntic cells contain a high concentration of carbonic anhydrase, and suggested that this enzyme has an important role in the mechanism of the formation of acid. However, Feldberg, Keilin & Mann (1940), using sulphanilamide, and Davenport (1946*b*), using thiophen-2-sulphonamide, both powerful inhibitors of carbonic anhydrase, failed to inhibit acid secretion. On the basis of these results, and some rough calculations, Davenport (1946*b*) believed that the necessity for the enzyme in the gastric mucosa had not been demonstrated. To do this it must be shown that, under conditions in the cell, the uncatalyzed uptake of CO_2 is slower than the observed rate, and that the amount of enzyme present is sufficient to produce the necessary acceleration of this reaction.

The calculations given in the Appendix show that the uncatalyzed rate of CO_2 uptake, to neutralize alkali within actively acid-secreting oxyntic cells, is too slow by a factor which depends on the extent to which the pH of the cell is alkaline to the equilibrium value. For example, in mammalian cells at a pH difference of 0·01, a magnification of 400-fold is required, whereas at a pH difference of 0·1 this is reduced to 40-fold. On the assumptions mentioned in the Appendix, the amount of carbonic anhydrase present in the oxyntic cells of cats is sufficient to produce a magnification of the order of 15,000-fold. This means that even if 99·7% of the enzyme were inhibited, the residual quantity could still produce a 40-fold magnification of the uncatalyzed CO_2 uptake. This may account for the results of Feldberg *et al.* (1940) and Davenport (1946*b*). A concentration of sulphonamide sufficient to produce 99·7% inhibition of the carbonic anhydrase of the gastric cells would necessarily have inhibited the enzyme of the red cells to a similar degree. As the red cells contain only about one fifth as much carbonic anhydrase per unit volume as the gastric cells, the carbon dioxide transport by the blood should have been so seriously affected as to lead to speedy death of the animal (cf. the calculations of Roughton, 1943). It would therefore seem that the gastric enzyme could not have been sufficiently inhibited in any published experiment.

SUMMARY

1. Intact tied tubes of frog and toad gastric mucosa secreted acid and mucus *in vitro* aerobically, and also produced an equivalent amount of alkali ($\pm < 1\%$), which was neutralized by CO_2 and passed into the nutrient solution as HCO_3^- ions.

2. No evidence of HCl secretion was obtained when sheets of sheep, frog or toad gastric mucosa were incubated in Warburg cups, although their respiration was enhanced by histamine. This is in agreement with the conception that HCl secretion into the lumen of the stomach is linked with an equivalent bicarbonate secretion into the nutrient solution.

3. The Q_{HCl} of active mucosae was often higher than the $|Q_{O_2}|$ or the respiratory Q_{CO_2}, and in these

cases external supplies of CO_2 were needed for the neutralization of alkali.

4. Calculations presented in the Appendix show that the uncatalyzed rate of CO_2 uptake, to neutralize alkali within very active oxyntic cells, is too slow by a factor of the order of 100, and make it probable that the necessary extra uptake can be readily produced by the carbonic anhydrase within oxyntic cells.

5. Anaerobically the tissue secreted neither acid nor mucus.

6. Evidence is presented which disproves the theories of HCl elaboration due to Bull & Gray (1945) and Conway *et al.* (1945).

I am greatly indebted to Mrs N. M. Bradford (née Longmuir) for assistance during part of these investigations, and to Prof. H. A. Krebs, F.R.S., for his encouragement, interest and advice in this study.

Appendix

By R. E. DAVIES and F. J. W. ROUGHTON, *Unit for Research in Cell Metabolism (Medical Research Council), Department of Biochemistry, The University, Sheffield, and Department of Colloid Science, The University, Cambridge*

The uncatalyzed rate of uptake of CO_2 *in oxyntic cells.* The value of the fraction, total volume of oxyntic cells/volume of acid secreting mucosa, is needed for the calculations. From the figures of Linderstrøm-Lang *et al.* (1935) it can be estimated that the fraction in pig mucosa, including the muscularis mucosa, is about 10%. Estimations on photographs of sections of human gastric mucosa varied from 8 to 11%, with an average just below 10%. Davenport (1939) found in cats that the average concentration of carbonic anhydrase was 39 Davenport units (E_D)/mg. wet weight in oxyntic cells and 2·7 E_D/mg. wet weight in acid-secreting mucosa. The surface cells contained small amounts, so the required fraction is not greater than 7%.

The following calculations are made for a mammalian mucosa with a Q_{HCl} of 50 μl./mg. dry wt./hr. at 38° containing 10% oxyntic cells, both well within the extremes. The output of CO_2 is given by the equation

$$\frac{d}{dt}[CO_2] = -k_u(1+l[B])[CO_2] + k_0(1+l[B])[H_2CO_3]$$

(Roughton, 1943)

in solutions with pH < 8. $[B]$ is the concentration of the proton acceptor in the buffer and l is its catalytic coefficient. Since $[HPO_4^{=}]$ is probably about 0·002 M inside the oxyntic cell and its l value is 8 at 0°, this term, and a correction for the protein buffers, can be neglected and the equation used in the form

$$\frac{d}{dt}[CO_2] = -k_u[CO_2] + k_0\frac{[a_H][HCO_3^-]f_{HCO_3}}{K_1}, \qquad (1)$$

where $$K_1 = \frac{[a_H][HCO_3^-]f_{HCO_3}}{[H_2CO_3]};$$

a_H = hydrogen ion activity, and

f_{HCO_3} = activity coefficient of HCO_3^- ion.

$[CO_2]$ is unlikely to be much more than 0·0011 M, and Irving (1932) gives the 'total CO_2' in dog fundic mucosa as about 30 ml./100 g. so $[HCO_3^-] = 0{\cdot}0134$ M.

Taking the ionic strength of blood as 0·13 (Hastings & Sendroy, 1925) and assuming similar conditions within oxyntic cells then

$$\log f_{HCO_3} = -0{\cdot}5\sqrt{0{\cdot}13} \text{ (cf. Roughton, 1935)}$$
$$= \bar{1}{\cdot}820$$

and $$f_{HCO_3} = 0{\cdot}66.$$

Roughton (1943) gives $k_0 = 89$ mol./l./sec., $k_u = 0{\cdot}131$ mol./l./sec., and $pK_1 = 3{\cdot}47$ at 38° at infinite dilution.

Substituting these values in equation (1), at equilibrium

$$\frac{d}{dt}[CO_2] = 0 = -(0{\cdot}131 \times 0{\cdot}0011) + \left(\frac{89 \times a_H \times 0{\cdot}0134 \times 0{\cdot}66}{10^{-3{\cdot}47}}\right),$$

therefore, $$pa_H = 7{\cdot}20_7.$$

If the pa_H of the cell is maintained below this value, CO_2 is evolved, whilst above it CO_2 is absorbed.

At 0·01 pa_H units on the alkaline side of equilibrium, application of equation (1) shows that

$$\frac{d}{dt}[CO_2] = -0{\cdot}03 \times 10^{-4} \text{ mol./l./sec.}$$

At 0·05 pa_H units above equilibrium,

$$\frac{d}{dt}[CO_2] = -0{\cdot}16 \times 10^{-4} \text{ mol./l./sec.}$$

At 0·10 pa_H units above equilibrium,

$$\frac{d}{dt}[CO_2] = -0{\cdot}30 \times 10^{-4} \text{ mol./l./sec.}$$

The oxyntic cells of the mucosa considered have a Q_{HCl} and a $Q_{HCO_3^-}$ of 500. Taking the dry weight as 0·2 wet weight, the usual value for mammalian mucosa, the rate of CO_2 uptake needed to produce this bicarbonate $\simeq 12 \times 10^{-4}$ mol./l./sec. The magnification of the CO_2 uptake required is therefore 400-fold at 0·01 pa_H, 75-fold at 0·05 pa_H, and 40-fold at 0·10 pa_H units on the alkaline side of equilibrium.

When the back reaction is neglected the required rate is 8·5 times the maximum uncatalyzed rate of CO_2 uptake. (In his calculations Davenport (1946*b*) neglected the back reaction and also used the low Q_{HCl} of about 11.)

In these calculations it is assumed that the whole volume of the cell is available to absorb CO_2 and that $[HCO_3^-]$ and $[CO_2]$ remain constant. When the cell is dependent upon a supply of CO_2 from arterial blood, diffusion limitations will reduce the steady state level of $[CO_2]$ below 0·0011 M. Thus these figures for the required magnification are minimal values and show the necessity for carbonic anhydrase in actively secreting oxyntic cells.

In frogs, while the Q_{HCl} values are lower by about 6-fold, the velocity constants for the hydration of CO_2 are lower by about 3·5-fold at 25·0°, so the enzyme is also required in frog gastric mucosa.

The effect of the carbonic anhydrase in oxyntic cells on the CO_2 uptake. The enzyme units used by Davenport (1939) were determined by the method of Meldrum & Roughton (1933) except that the temperature was 0° instead of 15°. One Davenport unit (E_D) is the amount of carbonic anhydrase which, when dissolved in 2 ml. of 0·1 M-Na_2HPO_4 in 0·1 M-KH_2PO_4 and mixed with 2 ml. of 0·2 M-$NaHCO_3$ in 0·02 M-NaOH, halves the time for the evolution of the second quarter of the total CO_2. If R = the reciprocal of the time with the catalyst, and R_0 = the reciprocal of the time without the catalyst, then there is one E_D unit in 4 ml. when

$$\frac{R-R_0}{R_0}=1.$$

To calculate the effect of enzymic activities, as expressed in Davenport units, on the rate of CO_2 uptake under the conditions prevailing in the gastric mucosa, it is first necessary to relate the Davenport unit to the more fundamental constants of carbonic anhydrase activity used by Roughton & Booth (1946).

According to these authors

$$R_{co}=\frac{k_{co}xE}{x+K_{mo}}, \qquad (2)$$

where R_{co} = the extra rate of CO_2 output in the presence of the enzyme,
k_{co} = velocity constant for dissociation of the enzyme-complex $E.H_2CO_3$,
$x=[H_2CO_3]$,
E = enzyme concentration, and
K_{mo} = Michaelis constant for CO_2 output.

Equation (2) is not convenient to work with, in view of the difficulty of calculating x exactly, but it may be transformed into a much more manageable form as follows.

Roughton & Booth (1946) have shown that

$$k_{co}=k_{cu} \quad \text{and} \quad K_{mo}=K_{mu}/K,$$

where k_{cu} = velocity constant for dissociation of the enzyme-complex $E.CO_2$,
K_{mu} = Michaelis constant for CO_2 uptake = 0·009 M at 0°,
K = equilibrium constant of the reaction

$$CO_2+H_2O \rightleftharpoons H_2CO_3,$$

i.e. $$K=\frac{[CO_2]}{[H_2CO_3]}=c.\ 900 \text{ at } 0°.$$

We then have

$$R_{co}=\frac{k_{co}xE}{x+K_{mo}}=\frac{k_{co}KxE}{Kx+KK_{mo}}=\frac{k_{cu}EC'}{C'+K_{mu}},$$

where $C'=Kx$ = the hypothetical concentration of dissolved CO_2 which would be in equilibrium with the $[H_2CO_3]$ at the stage used in Davenport's determination for the calculation of the enzyme units present.

This hypothetical or virtual $[CO_2]$ can be readily obtained if the CO_2-dissociation curve of the Meldrum-Roughton (M-R) mixture used by Davenport is known. The curve in question gives the relation between $[HCO_3^-]$ and $[CO_2]$ at complete equilibrium. It has been specially determined by us over the temperature range 0–38° (see Fig. 6 for results at 0°), by equilibrating 4 ml. of M-R mixture with CO_2 pressures varying from 0 to 200 mm. Hg in a modified Van Slyke constant-volume gasometric apparatus, and then determining the CO_2 contents of the gas and liquid phases. The usual 50 ml. Van Slyke gas chamber was replaced by a special 65 ml. chamber, which was the size of the boats used by Davenport. A more detailed account of the technique and experiments will be published elsewhere.

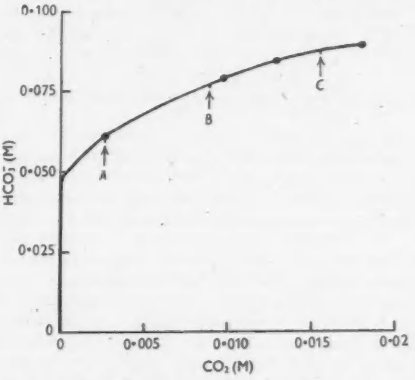

Fig. 6. CO_2 dissociation curve of the Meldrum-Roughton phosphate-bicarbonate mixture. The arrows at *A*, *B* and *C* indicate respectively the conditions at the start, at 3/8 completion and at the end of the reaction, when 4 ml. of M-R solution are shaken in a 65 ml. boat.

By this procedure we found that, at 0°, under the conditions prevailing in the boat experiments,

$[HCO_3^-]$ = 0·0878 M just after mixture,
= 0·0611 M at the end of reaction,
= 0·0778 M when the reaction is 3/8 completed, i.e. at the stage used for calculating the enzyme units present.

At the 3/8 stage, virtual $[CO_2]$ is the abscissa on Fig. 6, corresponding to 0·0778 M-HCO_3^-, and therefore = 0·0094 M.

$[HPO_4^{=}]$ = 0·07 M and pa_H = 7·24, by calculation.

With Davenport's conditions and 1 E_D enzyme unit in 4 ml., we then have

$$\frac{\left\{\frac{k_{cu}E\times 0\cdot0094}{(0\cdot0094+0\cdot009)}+k_u\times 0\cdot0094(1+l[HPO_4^{=}])\right\}-k_u\times 0\cdot0094(1+l[HPO_4^{=}])}{k_u\times 0\cdot0094\,(1+l[HPO_4^{=}])}=1 \text{ at } 0°.$$

Therefore $$\frac{k_{cu}E}{k_u\times 0\cdot0184\times 1\cdot56}=1.$$

Since $k_u = 0{\cdot}0021$, $k_{eu}E = 6 \times 10^{-5}$ mol. CO_2/l./sec. in the case of 1 Davenport unit.

In cat oxyntic cells there are 39 E_D/mg. wet weight, so 4 ml. of the cells contain $1{\cdot}56 \times 10^5$ E_D units, and therefore in the latter case

$$k_{eu} = 6 \times 10^{-5} \times 1{\cdot}56 \times 10^5 = 9{\cdot}4 \text{ at } 0°.$$

Within the oxyntic cell, taking $[CO_2]$ as 0·0011 M, and neglecting* the catalytic effects of the buffers for reasons given earlier, the magnification of the non-enzymic rate of CO_2 uptake at 0°

$$= \frac{k_{eu}E \times 0{\cdot}0011}{0{\cdot}0011 + K_{mu}} \div k_u \times 0{\cdot}0011$$

$$= \frac{9{\cdot}4}{0{\cdot}0021(0{\cdot}0011 + 0{\cdot}009)} = 440{,}000\text{-fold.}$$

This figure is greater than the magnification of $1{\cdot}56 \times 10^5$ for the M-R solution at 0° because (i) the substrate concentration in the latter case (i.e. 0·0094 M-virtual CO_2) is about the same as the value of the Michaelis constant, whereas in the former case the substrate concentration (i.e. 0·0011 M-CO_2) is only about one-eighth as great; (ii) the oxyntic cells are not known to contain inorganic catalysts corresponding to the $[HPO_4^=]$ in the M-R solution.

According to Kiese (1941), and to the rough preliminary data of Meldrum & Roughton (1933), the Michaelis constant increases about 3-fold per 10° rise, and should thus be about 50 to 60 times greater at 38° than at 0°. The substrate concentration, both in the M-R solution and in the case of 0·0011 M-CO_2 solution, is therefore very small compared with the value of the Michaelis constant at 38°; this means, if the effect of $[HPO_4^=]$ is neglected, that the magnification must be sensibly the same in the two cases and equal to $k'_{eu}E/k'_u K'_{mu}$, where symbols with primes denote the corresponding constants at 38°.

From these arguments it would follow that

$$\frac{\text{magnification in 0·0011 M-}CO_2\text{ at 0°}}{\text{magnification in 0·0011 M-}CO_2\text{ at 38°}} = 1{\cdot}8 \times \frac{\text{magnification in M-R solution at 0°}}{\text{magnification in M-R solution at 38°}}.$$

According to the recently amended data of Meldrum & Roughton (Roughton, 1948) on the enzymic and non-enzymic rates of CO_2 output from M-R solutions at temperatures ranging from 0·2° to 34·95°,

$$\frac{\text{magnification in M-R solution at 0°}}{\text{magnification in M-R solution at 38°}} = c.\ 17.$$

Assuming that the value of l for $HPO_4^=$ does not alter greatly from 0° to 38° (see below), the magnification in 0·0011 M-CO_2 at 38° should be $1/1{\cdot}8 \times 17 = 1/30$ times the magnification at 0°, so that for the conditions prevailing in the gastric mucosa, the magnification should be 440,000/30, i.e. about 15,000-fold. This figure is only about one fifth of the figure of 70,000-fold at 38° previously given by Davies, Longmuir & Crane (1947). The discrepancy is in part due to the fact that the figure, in the literature at the time, for the effect of temperature on the rate of the enzymic reaction was too high (Roughton, 1943), and in part to a theoretical error in the mode of calculation adopted. It is hoped that the latter is now reliable.

The concentration of carbonic anhydrase in frog gastric mucosa is about 0·1–0·2 E_D/mg. wet weight (Davenport, 1946*a*). Assuming that the concentrations of HCO_3^- and CO_2 are similar to those in the mammalian oxyntic cells, but that the temperature is 25° and the enzyme content 0·15 E_D/mg. wet weight, this concentration of enzyme could produce a magnification of about 1,200-fold.

Since Roughton (1943) states that the phosphate in the Meldrum-Roughton solution and the chloride in the red cell both inhibit carbonic anhydrase to about the same extent at the pH of red cells, no correction need be made for this effect in oxyntic cells.

The uncertainties in these calculations are (1) the extrapolation of the temperature coefficient of the magnification up to 38°, (2) the assumption of a linear relation between enzyme concentration and activity at high concentrations, (3) the possibility that the general environment of the enzyme in the cell may modify its activity, (4) the assumption that diffusion has no limiting effect on the reaction, and (5) the assumption that l is constant from 0° to 38°.

As regards (1) and (5), the uncertainty is probably now not large. In the case of these gastric cells we have no information in regard to (2) and (3), but unpublished calculations by Roughton, based on the rough data of Dirken & Mook (1930) on the rate of CO_2 reactions in red cell suspensions and strong haemoglobin solutions, suggest that the uncertainty may well be only of the order of 2- or 3-fold. The effect of diffusion is very difficult to assess until more exact knowledge is available as to the distances which the CO_2 has to diffuse from the capillaries to the highly concentrated enzyme in the cells.

Apart from the uncertainty as to what limiting effect is caused by diffusion, these calculations make it probable that the necessary extra uptake can be readily produced by the carbonic anhydrase within the oxyntic cells.

REFERENCES

Brücke, E. (1859). *S.B. Akad. Wiss. Wien*, **37**, 131.

Bull, H. B. & Gray, J. S. (1945). *Gastroent.* **4**, 175.

Conway, E. J., Fitzgerald, O. & Walls, D. (1945). *Nature, Lond.*, **156**, 477.

Davenport, H. W. (1939). *J. Physiol.* **97**, 32.

Davenport, H. W. (1943). *Gastroent.* **1**, 383.

Davenport, H. W. (1946*a*). Private communication.

Davenport, H. W. (1946*b*). *Physiol. Rev.* **26**, 560.

Davies, R. E. (1946). *Biochem. J.* **40**, xxxv.

Davies, R. E., Longmuir, N. M. & Crane, E. E. (1947). *Nature, Lond.*, **159**, 468.

Delrue, G. (1930). *Arch. int. Physiol.* **33**, 196.

Deutsch, W. & Raper, H. S. (1936). *J. Physiol.* **87**, 275.

Dirken, M. N. J. & Mook, H. W. (1930). *J. Physiol.* **73**, 273.

Dixon, M. (1943). *Manometric Methods*, 2nd ed. Cambridge: University Press.

Edkins, N. & Murray, M. M. (1924). *J. Physiol.* **59**, 271.
Feldberg, W., Keilin, D. & Mann, T. (1940). *Nature, Lond.*, **146**, 651.
Friedemann, T. E. & Graeser, J. B. (1933). *J. biol. Chem.* **100**, 291.
Friedman, M. H. F. (1943). *Proc. Soc. exp. Biol., N.Y.*, **54**, 42.
Glenn, P. M. (1946). *Gastroent.* **6**, 409.
Gray, J. S., Adkison, J. L. & Zelle, K. (1940). *Amer. J. Physiol.* **130**, 327.
Hanke, M. E. (1937). *Science*, **85**, 54.
Hanke, M. E., Johannesen, R. E. & Hanke, M. M. (1931). *Proc. Soc. exp. Biol., N.Y.*, **28**, 698.
Hastings, A. B. & Sendroy, J. (1925). *J. biol. Chem.* **66**, 445.
Hoerr, N. L. & Bensley, R. R. (1936). *Anat. Rec.* **65**, 417.
Hollander, F. (1943). *Gastroent.* **1**, 401.
Irving, L. (1932). *J. cell comp. Physiol.* **2**, 149.
Jones, H. Bence (1845). *Philos. Trans.* p. 335.
Keilin, D. (1929). *Proc. Roy. Soc.* B, **104**, 206.
Kiese, M. (1941). *Biochem. Z.* **307**, 400.
Krebs, H. A. (1933). *Hoppe-Seyl. Z.* **217**, 191.
Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
Kurtz, L. D. & Clark, B. B. (1946). *Fed. Proc.* **5**, 188.
Linderstrøm-Lang, K., Holter, H. & Ohlsen, A. S. (1935). *C.R. Lab., Carlsberg*, **20**, 66.
Lutwak-Mann, C. (1947). *Biochem. J.* **41**, 19.
McIver, M. A., Redfield, A. C. & Benedict, E. B. (1926). *Amer. J. Physiol.* **76**, 92.
Meldrum, N. U. & Roughton, F. J. W. (1933). *J. Physiol.* **80**, 113.
Ni, T. G. & Lim, R. K. S. (1928). *Chin. J. Physiol.* **2**, 45.
Peters, J. P. & Van Slyke, D. D. (1931). *Quantitative Clinical Chemistry*, **1**, *Interpretations*. London: Baillière, Tindall and Cox.
Rehm, W. S. & Enelow, A. J. (1945). *Amer. J. Physiol.* **144**, 701.
Rosenthal, O. & Lasnitzki, A. (1928). *Biochem. Z.* **196**, 340.
Roughton, F. J. W. (1935). *Physiol. Rev.* **15**, 241.
Roughton, F. J. W. (1943). *Harvey Lect.* **39**, 96.
Roughton, F. J. W. (1948). *J. Physiol.* **107**, 12P.
Roughton, F. J. W. & Booth, V. H. (1946). *Biochem. J.* **40**, 319.
Teorell, T. (1933). *Skand. Arch. Physiol.* **66**, 225.
Warburg, O. (1923). *Biochem. Z.* **142**, 317.
Warburg, O. (1926). *Biochem. Z.* **172**, 432.

Production of Ulcers in Isolated Frog Gastric Mucosa*

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Previous experiments (see Davies, 1948) have shown that when isolated frog gastric mucosa secretes acid, an equivalent amount of alkali is also produced. Normally this is neutralized by CO_2 and the resulting HCO_3^- ions are passed into the nutrient medium. The amount of HCl produced in highly active mucosae in bicarbonate saline gassed with 5% CO_2+95% O_2 (vol. % of gases throughout) was up to 100% above the metabolic CO_2 production and therefore external supplies of CO_2 were needed to neutralize this alkali. During these experiments (done in autumn, winter and early spring) it was found that the more active 'tied bags' of mucosa in phosphate saline gassed with 100% O_2 produced viscous whitish secretions containing much cell debris (indicating tissue damage), although in bicarbonate saline gassed with 5% CO_2+95% O_2 the secretions were clear and mobile.

The experiments described in this paper were designed to discover whether ulceration would occur when the experimental conditions interfered with the neutralization of the alkali by CO_2; this would lead to an accumulation of unneutralized alkali.

It was found that whilst mucosae with high secretory activities remained undamaged in bicarbonate saline, active mucosae were regularly ulcerated and even perforated in phosphate salines. Variations in the concentration of the phosphate buffer of the saline medium did not affect the occurrence of this ulceration.

EXPERIMENTAL

Saline media. The bicarbonate saline used for measurements of the balance of the O_2 uptake and the CO_2 output was the physiological salt solution of Krebs & Henseleit (1932) diluted by 0·29 vol. of water and gassed with 5% CO_2+95% O_2 or with 5% CO_2+95% N_2. During measurements of the O_2 uptake two alternative solutions were used. These were the phosphate saline of Krebs (1933) diluted with 0·29 vol. of water, and a saline solution similar to this phosphate saline, but containing only 0·0011 instead of 0·0084 M-phosphate. Both were gassed with 100% O_2. All salines contained 0·2% glucose.

Material. Frogs (*Rana temporaria temporaria* L.) were captured locally, the web tattooed with Indian ink for identification, and housed in a concrete froggery at 15–17°. An excess of cockroaches or worms was provided for food.

The gastric mucosa was washed, isolated and closed with silk thread at both ends (Davies, 1948). The amount of secretion produced and the total content of the mucosa

* A part of this work was communicated to the Biochemical Society (Davies & Longmuir, 1946).

after the experiment were determined by weighing as previously described. In all cases the preparations were carefully examined for ulcers and perforations. The secretions were classed as clear or cloudy by visual inspection. Microscopical observation showed that cloudiness was due to cells and cell debris.

Manometric procedure. The O_2 uptake, the resultant of O_2 uptake and CO_2 output, and the anaerobic glycolysis at 25·0° were measured with Warburg manometers in the usual way. Conical cups (20–30 ml. capacity) with a centre well were used, and histamine was added from the side arms to a final concentration of 5×10^{-5} M.

Estimation of the amount of acid secreted. In early experiments the previously described manometric method was used (Davies, 1948). In later experiments the acid in samples (10–100 mg.) of the gastric contents was estimated by electrometric titration with carbonate-free 0·01 N-NaOH in the apparatus shown in Fig. 1.

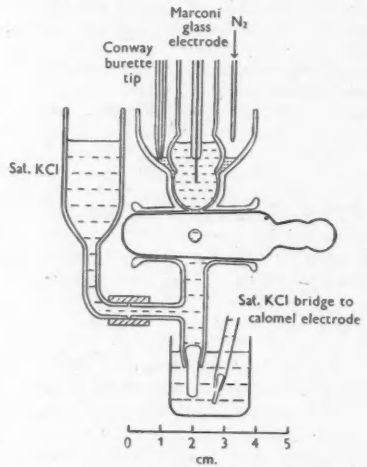


Fig. 1. Semi-micro electrometric titration unit.

A depression in a small glass bowl was ground to fit the thin bulb of a glass electrode (Marconi TM 388 A) so that even 5 mg. of secretion wetted this bulb evenly when it was placed in the depression. The alkali was added from a Conway burette and a jet of N_2 directed on to the surface of the liquid to keep away atmospheric CO_2 and to assist mixing. The electrode was carefully moved up and down after each addition of alkali to ensure complete reaction, which was delayed in many samples by the thick mucus present. A well made tap was fitted just below the depression in the bowl. A saturated solution of KCl leaking upwards round the barrel of this tap at an infinitesimal rate made electrical contact and gave no trouble due to diffusion potentials. A small reservoir of KCl solution maintained the necessary pressure and an internal ground-glass stopper in the tube below the tap prevented leakage downwards. The circuit was completed by a saturated-calomel electrode connected to a Marconi pH meter type TF 511C by means of a saturated KCl bridge of the type described by Crane, Davies & Longmuir (1948) one end of which dipped into a small beaker of saturated KCl solution which also contained the bottom tube of the titration unit. This apparatus has been used more than 150 times without damage to the electrode and has proved satisfactory and simple in operation.

Units. All Q_{HCl}, Q_{CO_2} and Q_{O_2} values in this paper are in μl./mg. dry wt./hr.

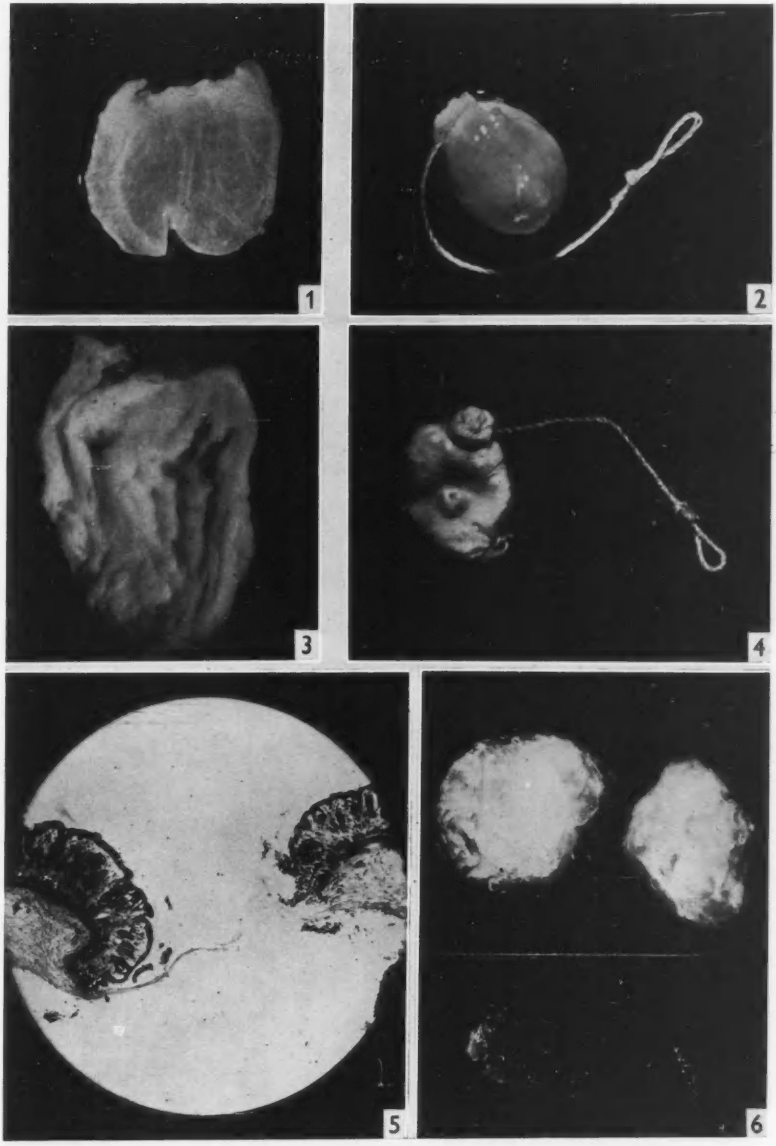
RESULTS

The method of comparing the rates of acid secretion in bicarbonate and in phosphate salines. In previous (winter) experiments (Davies, 1948) spontaneous acid secretion (a seasonal phenomenon) did not occur, and the mucosa produced acid only after stimulation with histamine. In this series of experiments carried out during late spring, summer and early autumn, some mucosae were found to be secreting acid as soon as measurements were taken after equilibration of the cups and manometers. A large overall uptake of CO_2 occurred in experiments in bicarbonate saline gassed with 5% CO_2 + 95% O_2 which, as has been shown previously (Davies, 1948), is indicative of the neutralization of alkali produced by the mucosal bag concomitantly with acid secretion. In experiments in phosphate saline gassed with 100% O_2, with alkali in the centre well of the cup, it was not possible to show that acid had been secreted until the tied mucosa was opened and its contents examined at the end of the incubation period.

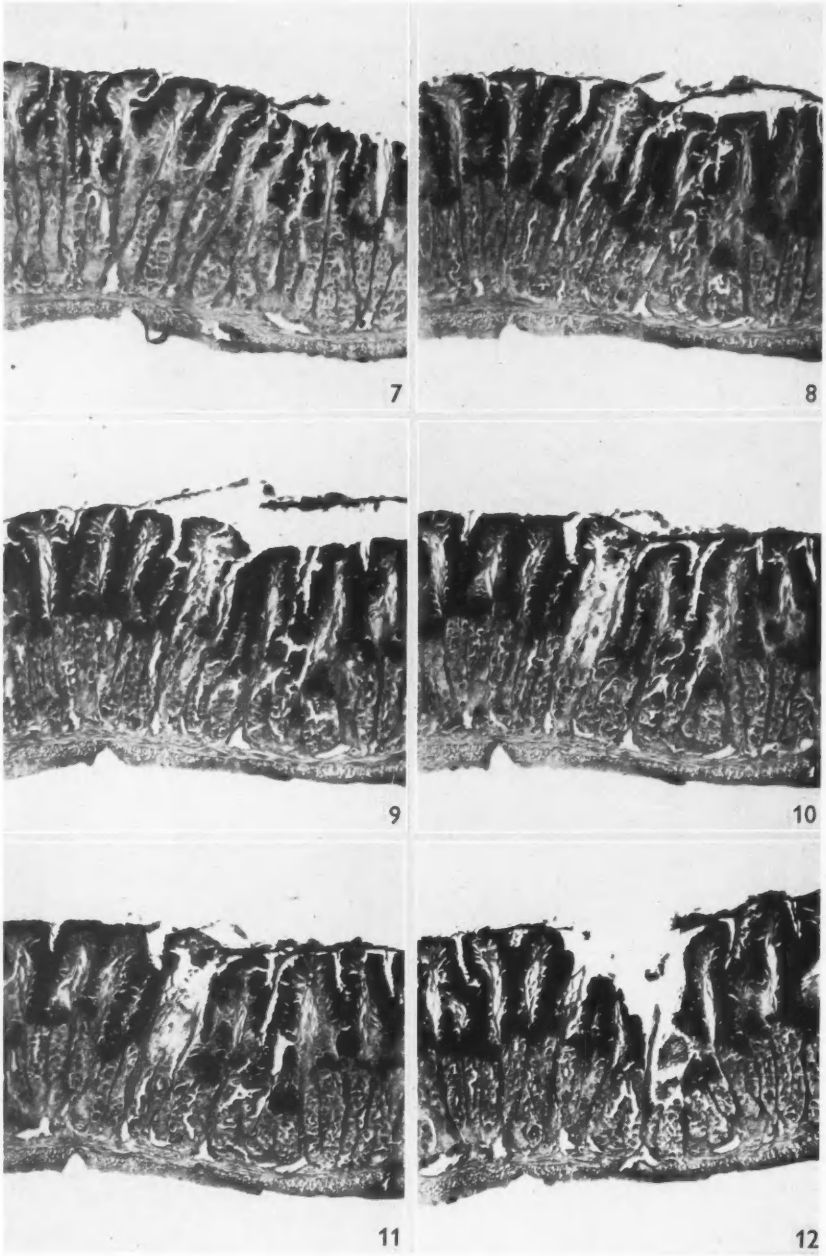
Spontaneous acid secretion in bicarbonate saline sometimes occurred not immediately but 1 or 2 hr. after the start of incubation and, since secretion might have begun in experiments in phosphate saline before stimulation with histamine, it is possible to calculate only an upper limit to the average Q_{HCl} of these mucosae during the period of stimulation. Therefore, in order to get comparable figures, the Q_{HCl} was calculated in all cases as an average for the total period of the experiment. Histamine was usually added 1 hr. after the start of incubation which usually lasted 4–6 hr. Frogs were selected at random and, on any given day, alternate mucosae were used in either bicarbonate or phosphate saline.

Experiments in phosphate salines. Table 1 shows clearly that a high average Q_{HCl} was associated with tissue damage and ulcer production in tied mucosae incubated in phosphate salines. Perforations occurred in actively acid-secreting mucosae whether the medium contained a low or a high concentration of phosphate buffer and, as the results were indistinguishable within experimental error, they have been grouped together.

Normal and ulcerated tissues are shown in Pls. 6 and 7. Mucosae with low average rates of acid secretion were not perforated, but the cloudiness of the mucus (Pl. 6) in some cases showed that some



R. E. DAVIES AND N. M. LONGMUIR—PRODUCTION OF ULCERS IN ISOLATED FROG GASTRIC MUCOSA



R. E. DAVIES AND N. M. LONGMUIR—PRODUCTION OF ULCERS IN ISOLATED FROG GASTRIC MUCOSA

Table 1. *Ulcer formation and average Q_{HCl} of tied mucosa in bicarbonate and phosphate salines*

(Total number of experiments 141. Nine tied mucosae under anaerobic conditions produced no acid, no mucus and were not damaged. No damage occurred in open sheets of mucosa in 17 experiments in bicarbonate saline and 15 experiments in phosphate saline.)

Average Q_{HCl} ... (μl./mg. dry wt./hr.)	Number of experiments in bicarbonate saline						Number of experiments in phosphate saline					
	No histamine			5×10^{-5} M-histamine			No histamine			5×10^{-5} M-histamine		
	<0·5	0·5→1·5	>1·5	<0·5	0·5→1·5	>1·5	<0·5	0·5→1·5	>1·5	<0·5	0·5→1·5	>1·5
Clear mucus, no damage	5	1	—	15	8	16	3	—	—	1	7	2
Cloudy mucus, small ulcers	—	—	—	—	—	1	3	1	—	5	9	4
Cloudy mucus, extensive necrosis and perforated ulcer	—	—	—	—	—	—	—	—	—	—	5	15

damage had occurred. Mucosae which secreted no acid were undamaged. The maximum average Q_{HCl} for the whole period of incubation was 2·8, and in no case was the average Q_{HCl} greater than the average $|Q_{O_2}|$ although the maximum Q_{HCl} may have risen above the maximum $|Q_{O_2}|$ in some cases. In the 54 tied mucosae incubated in phosphate salines the maximum $|Q_{O_2}|$ was from 1·7 to 3·9, average 2·7, standard deviation 0·50. All of 15 open sheets of mucosa were undamaged in phosphate saline. The $|Q_{O_2}|$ of some of these increased by 50–100% after the addition of histamine, an effect which, in tied tissue, is always associated with high rates of acid secretion.

Experiments in bicarbonate saline. Open sheets of tissue in bicarbonate saline were also undamaged in all of 17 cases. The results for 46 tied mucosae are shown in Table 1. The average Q_{HCl} had values between 0·0 and 5·4 and the maximum Q_{HCl} values estimated by the method of Davies (1948) were from 0·0 to 7·6. All but one of these mucosae were undamaged and the secretions were clear. The one damaged mucosa had the relatively high average Q_{HCl} of 3·7 and maximum Q_{HCl} of 5·1, and although the central regions of the tissue were undamaged and most of the secretion was clear and mobile, a spot of cloudy, opaque mucus was found in a fold of the tissue at the cardiac end of the mucosa, and beside it was a tiny ulcer.

Experiments under anaerobic conditions. In 9 experiments in bicarbonate saline gassed with 5% CO_2 + 95% N_2 the mucosae produced neither acid nor mucus after the addition of histamine and were undamaged.

The secretions of normal and ulcerated tissue. The secretions from ulcerated mucosae were always viscous and opaque and contained much cell debris (Pl. 6) which would be expected to have reacted with some of the acid. Semi-microelectrometric titration of the secretions with 0·01N-NaOH, using the apparatus described (Fig. 1), showed that this was the case. For an equivalent molarity the clear mobile, acid secretions had a lower pH than the cloudy and were relatively less buffered (Figs. 2–4). All neutral secretions were thick with mucus. The cloudy secretions, due to their content of broken-down cells in addition to the mucus normally present, were more strongly buffered (Figs. 5–7). The theoretical curves are given for comparison on the assumption that the secretion was a pure unbuffered HCl solution of equivalent strength titrated to pH 7·4 (the pH of the saline media) with 0·01N-NaOH. Since the acid was usually more concentrated than this alkali, the curve of ml. alkali against pH of reaction mixture shows a point of inflexion between pH 1 and 3. At any pH value, the deviations of the slopes of the experimental and 'unbuffered' curves give a measure of the buffer capacity of the secretions. All the samples were buffered in the

alkaline region due to the presence of mucus, but only the secretions containing cell debris from mucosae incubated in phosphate salines were strongly buffered at low pH values.

can occur in isolated tied bags of frog gastric mucosa with high rates of HCl production. Such damage did not occur in either tied mucosae not secreting acid or with a low Q_{HCl}, or in open sheets of tissue. This

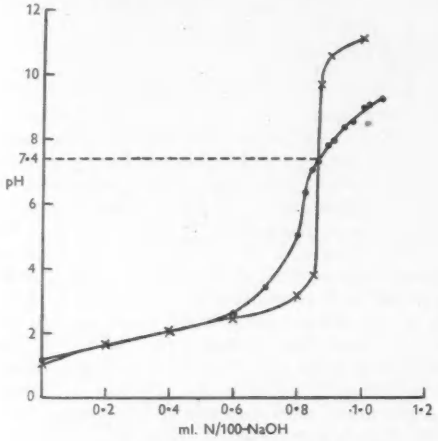


Fig. 2. 100·8 mg. gastric acid.

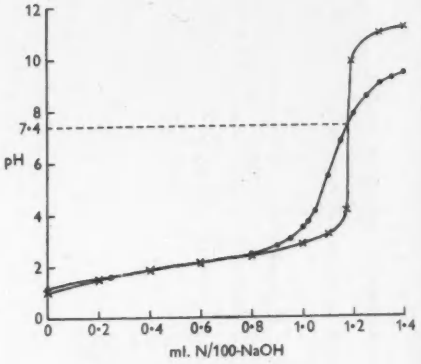


Fig. 3. 118·1 mg. gastric acid.

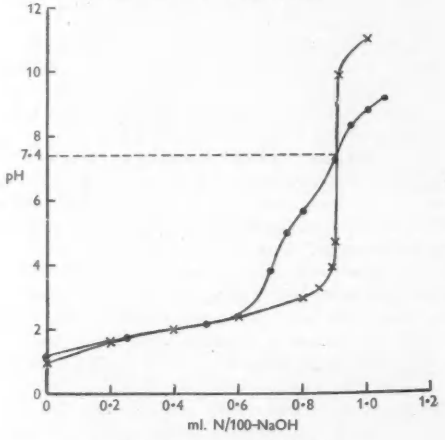


Fig. 4. 86·8 mg. gastric acid.

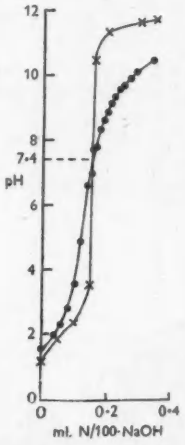


Fig. 5. 20·3 mg. gastric acid.

Figs. 2–7. Electrometric titration (dots) of samples of gastric acid with 0·01 N-NaOH to pH 7·4. The theoretical curves (crosses) were drawn on the assumption that the gastric contents were pure HCl solutions. For further details see Table 2.

DISCUSSION

Ulcer formation in the presence and absence of external supplies of CO_2

The experiments recorded in this paper show that, in the absence of the normal CO_2:HCO_3^- buffer in the medium, extensive tissue damage and perforation could be predicted from previous results (Davies, 1948) which showed that the oxyntic cells of an actively secreting mucosa required amounts of CO_2 in excess of the total metabolic CO_2 production of even the whole of the tissue. If this alkali were not neutralized then local damage would be expected. Tied mucosae in phosphate salines with a Q_{HCl} less

than their respiratory Q_{CO_2} would be undamaged if all the CO_2 were available to the oxyntic cells and none absorbed by the alkali in the centre well of the Warburg cup. With a rate of HCl production greater than the rate at which the CO_2 produced by

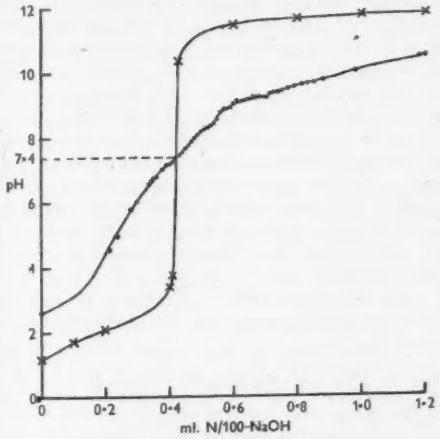


Fig. 6. 62·8 mg. gastric acid.

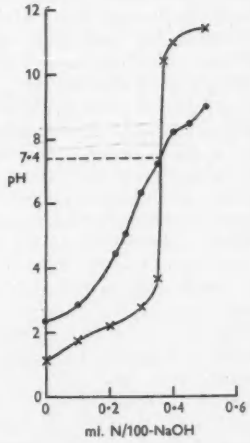


Fig. 7. 47·9 mg. gastric acid.

the mucosa could be absorbed by the oxyntic cells, damage must occur if other buffers are ineffective. It is of interest that Delrue (1933) and Gray & Adkison (1941) found lowered rates of acid secretion in isolated frog gastric mucosa when the HCO_3^- ion was omitted from the medium.

For reasons given earlier the present techniques preclude an estimate of the maximum Q_{HCl} of tied

Table 2. *Details of the experiments relating to Figs. 2–7*

(Tied tubes of mucosa were placed in 4 ml. nutrient phosphate saline gassed with O_2, or nutrient bicarbonate saline gassed with 5 % CO_2 + 95 % O_2. The tubes were weighed before and after incubation and after emptying. The differences between the increase in weight of the tube, the 'secretion' and the actual 'contents' are probably accounted for by extrusion of mucus (and cellular debris) into the lumen of the mucosa. Histamine to a final concentration of 5×10^{-5} M was added at the times indicated.)

Corresponding Fig.	Type of saline	Dry wt. of tissue (mg.)	Wet wt. of secretion (mg.)	Wet wt. of contents (mg.)	Consistency of contents	State of mucosa	HCl produced (µl.)	Molarity of H^+ in secretion	Molarity of H^+ in contents	Av. Q_{HCl} total period (µl./mg. dry wt./hr.)	Max. Q_{HCl} (µl./mg. dry wt./hr.)	Av. Q_{O_2} (µl./mg. dry wt./hr.)	Duration of exp. (hr.)	Time of addition of histamine (hr.)
2	Bicarbonate	39·8	268	283	Clear liquid	Undamaged	544	0·091	0·086	2·7	4·9	—	5·00	1·00
3	Bicarbonate	34·6	173	188	Clear liquid	Undamaged	421	0·109	0·100	2·8	4·5	—	4·20	1·00
4	Bicarbonate	17·9	124	130	Clear liquid	Undamaged	304	0·109	0·104	4·1	6·6	—	4·10	1·00
5	Phosphate	21·5	81	91	Fairly cloudy liquid	1 small ulcer, no perforations	156	0·086	0·077	1·5	—	−1·9	4·50	1·00
6	Phosphate	30·4	149	159	Cloudy liquid	6 small ulcers, no perforations	238	0·071	0·067	1·4	—	−2·3	5·30	1·30
7	Phosphate	25·9	147	174	Whitish viscous paste	1 large perforated ulcer	293	0·089	0·075	1·6	—	−2·1	7·00	1·30

mucosae in phosphate salines. However, the relation between the average Q_{HCl} and tissue damage (Table 1) shows that this damage was associated with high rates of secretion, was less severe with low rates and did not occur in mucosae which failed to secrete acid or gave only slight responses. Despite presumptive evidence of high rates of acid secretion in open sheets of mucosa (large increase in $|Q_{O_2}|$ after addition of histamine) none was damaged. This is because small amounts of CO_2 and HCO_3^- are always present in the phosphate salines during the incubation, and the CO_2 liberated by the acid is thus available to neutralize the alkali in the oxyntic cells and thus reform the HCO_3^- again.

The observation that salines with a high content of phosphate buffer failed to prevent ulceration in actively secreting tied mucosae is evidence that the alkali produced in the oxyntic cells is normally neutralized inside them. This is supported by the known high content of carbonic anhydrase in mammalian oxyntic cells and in frog gastric mucosa (Davenport, 1946), and by calculations (Davies, 1948) showing the necessity for this enzyme in active oxyntic cells. The cytoplasmic buffers may well play an intermediary part in this neutralization, but the overall effect is that the hydroxyl ions are converted quantitatively by CO_2 to bicarbonate and a trace of carbonate at the pH of the medium.

The fact that CO_2 can enter the oxyntic cell without an electric charge, and is then extruded as the negatively charged HCO_3^- ion, may well account for the superiority of the bicarbonate over the phosphate buffer system.

In all but 1 of 46 tied mucosae in bicarbonate saline no damage occurred and the secretions were clear. This mucosa was slightly damaged near a thick fold at the cardiac end. In relation to this case it is of interest to calculate the diffusion limitations on the absorption of CO_2.

Diffusion limitations to the rate of absorption of external supplies of CO_2

Krogh (1919), on the basis of a single determination, found that the diffusion constant of CO_2 was 35·7 times that of O_2 which he gave as 0·14 in muscle at 20°. This is the number of ml. (at 0° and 760 mm.) penetrating through a membrane 0·001 mm. thickness and 1 sq.cm. surface/min. when the pressure difference across the membrane is 1 atm. If the increase of the diffusion constant of CO_2 with temperature is similar to that of O_2 (Krogh, 1919) it will be 5·3 ml. CO_2 (at N.T.P.)/sq.cm./min. at 25° with a pressure gradient of 1 atm./0·001 mm. Warburg (1923) showed that

$$d' = \sqrt{[8C_0\,(D/A)]},$$

where C_0 is the pressure of gas outside the tissue in atm., A is the rate of utilization of the gas in ml./min./ml. tissue, D is the diffusion constant of the gas in ml. gas (N.T.P.)/sq.cm./min. with a pressure gradient of 1 atm./cm. and d' is the thickness of the sheet of tissue in cm. at which the gas concentration at the centre is just zero. Applying this formula to the uptake of CO_2 in tied frog gastric mucosa, where the CO_2 at 5% of 1 atm. pressure can diffuse in from one side only, and taking a dry weight/wet weight ratio of 0·2, we obtain for a tied mucosa 0·03 cm. thick $Q_{CO_2} = -17{\cdot}5$. If a fold makes the tissue 0·09 cm. thick, then $Q_{CO_2} = -2{\cdot}0$. These figures give amounts by which the Q_{HCl} can rise above $|Q_{O_2}|$ before diffusion limitations set in and are only approximate as the tissue has been assumed to be uniform. They account for the slight damage occurring at a fold of an active mucosa in bicarbonate saline and show that it is important to tie the silk loops, which close the tube of mucosa at the oesophageal and pyloric ends, at points well away from the corpus of the mucosa to avoid wrinkles in the tissue at the places where the acid is formed and where the CO_2 absorption takes place.

Ulcer formation and treatment

The ulcers shown in Pls. 6 and 7 involve more of the tissue than the oxyntic cells. It seems probable that, once local necrosis has started, the acid already secreted by other cells would extend the damage to other regions of the mucosa. This may be the case in the serial sections in Pl. 7, which show the 'growing point' of an ulcer extending through the tissue.

It is generally agreed that the oesophagus of the frog secretes pepsin, but there has been controversy as to whether the gastric mucosa secretes none at all (Lim, 1922) or small amounts (Friedman, 1937). Since the oesophagus was removed from our mucosae, it seems likely that HCl alone played the most important part in extending the tissue damage.

The significance of these results for the causation of human ulcers is not known. Most chronic human ulcers are formed in regions distant from the site of acid formation (Bockus, 1943), but a combination of factors causing stimulation of active acid secretion, but not providing sufficient CO_2 to neutralize the alkali formed in the oxyntic cells, may be operative in the causation of some types of acute human gastric ulcers. Calculations (Davies, 1948) show that the carbonic anhydrase normally present in oxyntic cells is necessary to facilitate the uptake of CO_2. Therefore, if this enzyme be absent or completely inactivated, tissue damage would be expected to follow on active acid secretion.

Davenport (1946) has recorded the presence of carbonic anhydrase in duodenal mucosa, and it is interesting to speculate whether this tissue acts rather like gastric mucosa in reverse, requiring supplies of CO_2 to secrete $NaHCO_3$ into the lumen of the duodenum and HCl into the blood. If this be

true it might throw light on the important experimental results of Cummins, Grossman & Ivy (1947) who found that duodenal ulcers occurred in dogs into whose stomachs physiological concentrations of HCl were dripped. These dogs all developed severe acidosis. However if 0·16N-$NaHCO_3$ was introduced by continuous drip, either by terminal ileostomy or intravenously, in quantities sufficient to maintain a normal blood pH, ulcers did not occur.

The results described in this paper, together with those of Cummins *et al.* (1947), suggest that the ideal antacid for the prevention of ulceration should evolve CO_2 in the presence of acid and should contain cations which do not upset the acid-base balance of the blood. The carbonate or bicarbonate of a basic protein such as a protamine, and milk saturated with CO_2, are therefore put forward as worthy of clinical trial.

SUMMARY

1. In the absence of external supplies of CO_2 (normally required to neutralize the alkali formed concomitantly with the acid), ulcerations and perforations occurred in tied bags of isolated frog gastric mucosa provided that the rate of acid secretion was high. Tied mucosae with low rates of secretion showed little or no ulceration.

2. Tied mucosae which failed to secrete acid, and open sheets of mucosa, suffered no damage.

3. Anaerobically, when mucosae produce neither acid nor mucus, no damage was observed.

4. In the presence of adequate supplies of CO_2, tied mucosae in bicarbonate saline were undamaged.

5. In the absence of external supplies of CO_2 the average Q_{HCl} was never greater than the average $|Q_{O_2}|$. In the presence of 5% CO_2 some mucosae had an average Q_{HCl} greater than the highest $|Q_{O_2}|$ found and a maximum Q_{HCl} about twice the value of this $|Q_{O_2}|$.

6. Ulcers occur if the alkali formed by oxyntic cells remains unneutralized.

The authors wish to record their appreciation to Mr J. Kugler of the Anatomy Department, University of Sheffield, for technical assistance with the histological work and to thank Prof. H. A. Krebs, F.R.S., for his encouragement and interest in this study and Dr H. E. Harding, Senior Lecturer in Pathology, University of Sheffield, for advice on histological and pathological matters.

REFERENCES

Bockus, H. L. (1943). *Gastroenterology.* **1.** Philadelphia: Saunders.
Crane, E. E., Davies, R. E. & Longmuir, N. M. (1948). *Biochem. J.* **43.** (In the Press.)
Cummins, G. M., Grossman, M. I. & Ivy, A. C. (1947). *Fed. Proc.* **6**, 93.
Davenport, H. W. (1946). *Physiol. Rev.* **26**, 560.
Davies, R. E. (1948). *Biochem. J.* **42**, 609.
Davies, R. E. & Longmuir, N. M. (1946). *Biochem. J.* **40**, lxiv.
Delrue, G. (1933). *Arch. int. Physiol.* **36**, 129.
Friedman, M. H. F. (1937). *J. cell. comp. Physiol.* **10**, 37.
Gray, J. S. & Adkison, J. L. (1941). *Amer. J. Physiol.* **134**, 27.
Krebs, H. A. (1933). *Hoppe-Seyl. Z.* **217**, 191.
Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
Krogh, A. (1919). *J. Physiol.* **52**, 391.
Lim, R. K. S. (1922). *Quart. J. micr. Sci.* **66**, 187.
McManus, J. F. A. (1946). *Nature, Lond.*, **158**, 202.
Warburg, O. (1923). *Biochem. Z.* **142**, 317.

EXPLANATION OF PLATES

PLATE 6

Fig. 1. Appearance of normal frog gastric mucosa opened along the greater curvature (cf. ulcerated mucosa, Fig. 3). ×2·3.

Fig. 2. Normal distended tied bag of mucosa after 5 hr. stimulation with 5×10^{-5}M-histamine in bicarbonate saline. The bag was flat and empty at the start of the experiment. Av. Q_{HCl} 3·0, max. Q_{HCl} 4·3. ×2·3.

Fig. 3. Extensive ulcerations in a bag of mucosa (opened along the lesser curvature; cf. Fig. 1). 5 hr. stimulation with 5×10^{-5}M-histamine in phosphate saline. Av. Q_{HCl} 1·9, av. Q_{O_2} −2·6. ×3·7.

Fig. 4. Perforations in mucosa after 5 hr. stimulation with 5×10^{-5}M-histamine in phosphate saline (cf. Fig. 2). Av. Q_{HCl} 1·6, av. Q_{O_2} −2·0. ×2·3.

Fig. 5. Perforation in mucosa after 4 hr. stimulation with 5×10^{-5}M-histamine in phosphate saline containing only 0·0011M-phosphate. Only a thread of submucosal tissue remains. Av. Q_{HCl} 2·1, av. Q_{O_2} −2·9. 5μ. section, fixed with ethanolic Bouin, stained with periodic acid and Schiff's reagent (McManus, 1946) and haematoxylin. ×55.

Fig. 6. Samples of clear and cloudy secretions from normal and perforated mucosae respectively. The cloudiness is caused by the high content of cells and cell debris. ×2·3.

PLATE 7

Figs. 7–12. 5μ. sections of ulcerated mucosa. Figs. 7–11 are serial, but three sections omitted between Figs. 11 and 12. This series shows the extension of ulceration through the tissue. Dissolution of cells starts below the surface epithelium in the distal third of the gland and rapidly involves most of the cells in the distal two thirds. Ulceration occurs a little later by shedding of the surface mucous cells. 5 hr. stimulation with 5×10^{-5}M-histamine in phosphate saline containing only 0·0011M-phosphate. Av. Q_{HCl} 1·8, av. Q_{O_2} −2·5. Fixed and stained as Pl. 6. ×150.

The Isomeric Transformation of Urea into Ammonium Cyanate in Aqueous Solutions

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Walker & Hambly (1895) showed that the isomeric transformation of ammonium cyanate into urea (Wöhler, 1828) was spontaneously reversible in aqueous solutions. At equilibrium at 100°, a 0·1M-solution of urea was said to contain approximately 95% urea and 5% ammonium cyanate. Werner (1923) denied formation of cyanate from urea at temperatures below 60°. According to this author aqueous solutions of urea, kept for many months at room or body temperature under sterile conditions, did not contain traces of cyanate.

Since some pharmacological actions of cyanate were recently described (Schütz, 1945, 1946; Birch & Schütz, 1946), the question whether cyanate is formed in the mammalian organism was investigated. As the first step in studying this question, experiments were carried out with the sole aim of establishing whether cyanate was spontaneously formed in aqueous solutions of urea, at body or room temperature. Experiments dealing with living tissues will be reported separately.

The isomeric change in question could, theoretically, be demonstrated by showing a gradual decrease in the amount of urea originally present in the solution. Since, however, even at high temperatures, only a small percentage of the urea originally present is known to undergo the isomeric change into NH_4CNO, the expected decrease would probably be within the margin of error of the most sensitive quantitative methods for the determination of urea. On the other hand, the existing methods for the detection and quantitative determination of cyanate (Walker & Hambly, 1895; Hertig, 1901) were found to be too insensitive, especially when urea was present in higher concentrations.

In this paper two new methods are described for the qualitative and quantitative determination of cyanate in aqueous solutions. By means of these procedures evidence was obtained for the spontaneous formation of cyanate in aqueous solutions of urea, at body and room temperature.

METHODS AND EXPERIMENTAL

Spectroscopic method

Materials

Methaemoglobin. Human red cells from citrated blood were washed three times with 0·9% (w/v) NaCl solution. The white cells were removed and the red cells were lysed by adding 6 volumes of distilled water. ·5% (v/v) of a freshly prepared saturated solution of $K_3Fe(CN)_6$ was added. The solution was left standing for 30 min. at room temperature, and was dialyzed overnight against running tap water through parchment paper, to remove surplus reagent. Thereafter the solution was centrifuged and buffered (10% v/v) with 0·1M-phosphate buffer (pH 7·3). Only fresh solutions were used.

Urea was recrystallized from ethanol. Sterile solutions were made by dissolving the weighed amount in ethanol; the solution was transferred into a sterile flask, closed with cotton wool, and the solvent removed by placing in a dry oven (100°). After cooling to room temperature, the desired amount of distilled water was added, sterile conditions being maintained throughout. The same results concerning the formation of cyanate were obtained, however, if the growth of micro-organisms was inhibited simply by saturating the solutions with chloroform or toluene.

Spectroscopy

A Hartridge reversion spectroscope was used in a dark room. If previously incubated, the solutions were brought to room temperature before the readings. A concentrated solution of methaemoglobin (metHb) was added drop by drop thereafter until the α-band became just clearly visible. Since the shift depended on the ratio CNO-metHb/metHb, care was taken not to add an undue excess of metHb to the urea solutions. For the readings, the solutions were transferred into containers of *c.* 2 cm. width. Samples which contained much carbonate, formed through decomposition of cyanate, were brought to pH 7·4 by a suitable addition of 0·4M-phosphate buffer.

Cyanate was found to combine readily with metHb in neutral solutions (Schütz, 1945). On saturation with cyanate the α-band of the absorption spectrum of metHb is shifted from 6325 to 6285 A. This shift can only be observed by means of small dispersion spectroscopes. Since freshly prepared solutions of urea did not alter the spectrum of metHb, the method consists in the determination of the position of the α-band after the addition of metHb solution to the urea solution. Details of the reaction of cyanate with metHb, and with other haemoglobin derivatives, will be reported in a future communication. The method described here is not applicable, without alteration, to solutions containing serum albumin or other substances which also combine with metHb.

Experimental

In preliminary experiments it was found that freshly prepared solutions of urea did not cause the slightest shift of the α-band of metHb added as described above. Neither did the buffer solution, nor

the chloroform nor toluene added as a bacteriostatic, cause any change in the position of the α-band.

It was found quite unsuitable to incubate the urea solutions with metHb, not only because the latter quickly coagulates, but also on account of its action as a cyanate-fixing substance. If left in the urea solution for hours, it 'traps' cyanate, thus shifting the equilibrium point of the urea $\rightleftharpoons NH_4CNO$ equilibrium in favour of NH_4CNO. The urea solutions were, therefore, incubated, with or without buffer, either sterile or saturated with a bacteriostatic. At intervals, samples were taken, cooled, and readings made immediately after the addition of metHb. Typical results are shown in Table 1.

at lower temperatures, is needed to show the presence of cyanate. The lowest concentration of cyanate which can be detected in this way was found to be of the order of 2×10^{-4}M. No quantitative assessment, however, was made with this method, since the manometric method, described below, was found to be superior for this purpose.

Since ammonium cyanate is formed from urea, control experiments were made on the possible action of ammonium ions on metHb, and on the reaction of cyanate with metHb. Under the conditions, and for the short periods, of the experiments shown in Table 1, no influence on either was found by ammonium chloride present in concentrations up

Table 1. *The position of the α-band of methaemoglobin immediately after its addition to fresh or incubated aqueous solutions of urea*

(α-Band of metHb, 6325 A.; α-band of cyanate-metHb, 6285 A.)

MetHb (2–3 drops) added at room temperature to	α-Band (average of several readings) (A.)
1·8M-Urea in sterile water, freshly prepared	6324 (±4)
1·8M-Urea in sterile water, after 41 hr. at 38°	6285 (±3)
1·8M-Urea in water, saturated with chloroform, freshly prepared	6325 (±5)
1·8M-Urea in water, after 41 hr. at 38°	6288 (±3)
1·8M-Urea in water, saturated with toluene, freshly prepared	6324 (±5)
1·8M-Urea in water, after 41 hr. at 38°	6289 (±4)
1·8M-Urea in 0·4M-phosphate buffer (pH 7·4), sterile, freshly prepared	6323 (±3)
Same solution, after 41 hr. at 38°	6285 (±2)
Same solution, after 41 hr. at room temperature	6318 (±4)
Same solution, after 5 days at room temperature	6308 (±3)
Same solution, after 41 hr. at 4°	6322 (±4)
Same solution, after 5 days at 4°	6322 (±5)
Controls	
Sterile water	6325 (±4)
0·05M-NaCNO, in water	6285 (±2)
Water saturated with chloroform, after 41 hr. at 38°	6326 (±5)
Water saturated with toluene, after 41 hr. at 38°	6325 (±4)
0·4M-Phosphate buffer (pH 7·4), sterile	6324 (±4)
0·4M-Phosphate buffer (pH 7·4), after 42 hr. at 38°	6325 (±3)

The figures show that with increasing time, and concentration of urea, the α-band of added metHb is shifted increasingly towards the point characteristic for CNO-metHb. Since the relevant shift was always very clearly observed with pure aqueous solutions of urea after incubation, the shift cannot conceivably be due to any other substance than cyanate. Moreover, when the maximum shift was observed, the α-band was situated as in the case of pure CNO-metHb.

It was also noted that the transformation, especially at the lower temperatures, is a very slow process. At least 6 hr. incubation at 38°, and more to 5% of the corresponding concentration of urea. Ammonium ions, in this range of concentration, had no influence on this qualitative method of detecting cyanate if the pH was maintained within the range 7·0–7·4.

Manometric method

Materials

Urea solutions were freshly prepared for each experiment and saturated with chloroform. Traces of the latter substance did not interfere with the determinations.

Sodium cyanate. This was made from urea according to Bader, Dupré & Schütz (1948). A pure sample of sodium cyanate was provided by Glaxo Laboratories Ltd.

It is known that cyanate decomposes on addition of acids into CO_2 and NH_3. This was studied with the manometric technique of Warburg. The solution of NaCNO was placed in the main compartment, and buffer solutions of different pH in the side arm. After equilibration at room temperature, the buffer was added to the NaCNO solution, and the CO_2 evolved was measured. The results are given in Fig. 1. It can be seen that only at pH < 5 does the decomposition of cyanate become obvious; only in very acid solutions does it decompose rapidly. Contrary to the common belief, cyanate was found to be relatively stable in moderately acid solutions (pH > 5).

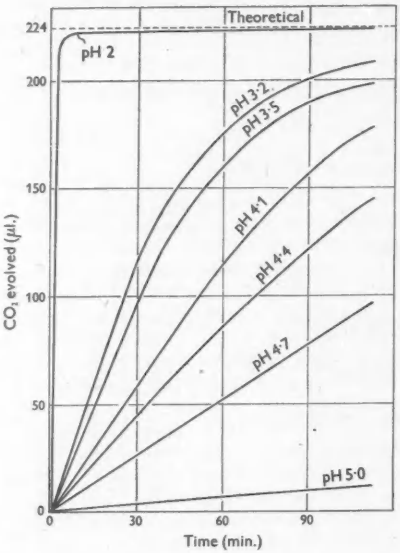

Fig. 1. Decomposition of sodium cyanate in aqueous solution at room temperature and various pH values.

In these experiments NaCNO was preferred to NH_4CNO because of the partial conversion of the latter into urea, even at room temperature. If NH_4Cl was added to NaCNO in equimolar amounts the same results were obtained as with NaCNO alone if the solution was not older than 0·5 hr. Similar results were obtained with different concentrations of NaCNO.

Since cyanate was found to be relatively stable at pH 5·0, it was possible to adopt the following procedure. Any carbonate or bicarbonate present was eliminated by the addition from one side arm of a buffer of pH 5·0. Within the time needed to re-establish equilibrium the decomposition of cyanate was found to be negligible at this pH and at room temperature. Then, after renewed equilibration, an appropriate amount of a strong acid was added from the second side arm, by which the pH of the mixture of cyanate and buffer was brought to 2, or less. The cyanate rapidly decomposed, and the corresponding amount of CO_2 evolved was measured.

Since the amount of CO_2 evolved after addition of the buffer solution (pH 5) corresponds to the carbonate and bicarbonate present in the solution, while that evolved after the addition of the strong acid measured the cyanate present in the mixture, carbonate and cyanate can be determined in the same sample and at the same time. This is useful since cyanate is known to decompose into carbonate to a varying degree according to the prevailing conditions.

Experimental

Incubated samples of solutions of urea were brought to room temperature, and 1–3 ml. of these transferred into the main compartment of a two-armed Warburg vessel, 0·2–0·3 ml. of 3M-acetate buffer being placed in one, and 0·2–0·3 ml. of 10% (w/v) H_2SO_4 in the other side bulb. After equilibration, the buffer was first added and, after re-establishment of equilibrium and noting of the pressure changes, the H_2SO_4 was added from the other bulb, and the CO_2 evolved again recorded.

Typical results are shown in Table 2. With known quantities of NaCNO, the results are in good agreement with the theoretical amounts, in the presence or absence of urea or phosphate buffer or both. It can further be seen that practically no CO_2 was evolved, after addition of the acid to a blank, which consisted of a freshly prepared solution of urea. This indicates that cyanate is not formed rapidly. The small pressure changes observed in this, and buffered blanks, are to be ascribed mainly to the altered CO_2 solubility and the small changes in vapour pressure which occur when the acid is added to the mixture of buffer and urea solution. These blank values are, however, so small in comparison with the data obtained with the same solutions after incubation, that only a very small correction factor would be needed.

Obviously the method is suitable to determine how much cyanate is formed in pure aqueous, or buffered, solutions of urea, since none of the substances present in such solutions interferes with the procedure; neither was any indication obtained of formation of cyanate from urea through manipulations required by the method.

Application of this method to biological material would yield results difficult to interpret, since there might be other substances present, besides cyanate, which would be stable at pH 5 and from which CO_2 would be liberated at pH 2.

With certain limitations, it can be applied, however, to mixtures of biological material and added NaCNO, more concentrated acetate buffer (3M) being required, and a higher concentration of H_2SO_4 to overcome the buffering capacity of biological materials. Results cited in this paper were also obtainable with these higher concentrations of buffer and acid.

Table 2. CO_2 *evolved from solutions after addition of acetate buffer* (pH 5), *showing amount of bicarbonate and carbonate*

(If cyanate is present subsequent addition of H_2SO_4 to the mixture causes a further evolution of CO_2 due to acid decomposition of cyanate.)

	CO_2 evolved (μl.)			
	After 0·3 ml. 3M-acetate buffer (carbonate, etc.)		After further addition of 0·3 ml. 10% H_2SO_4 (cyanate)	
	Found	Theoretical	Found	Theoretical
2M-Urea (fresh), 2 ml.	+ 16	—	+ 3	—
2M-Urea (fresh), 1 ml. 0·1M-Phosphate buffer, pH 7·4, 1 ml.	− 7	—	+ 12	—
0·1M-Phosphate buffer, pH 7·4, 2 ml.	− 7	—	+ 14	—
0·01M-NaCNO, 1 ml.	+ 9	—	+210	+224
0·01M-NaCNO, 1 ml. 0·1M-Phosphate buffer, pH 7·4, 1 ml.	− 6	—	+196	+224
0·01M-NaCNO, 1 ml. 0·1M-Phosphate buffer, pH 7·4, 1 ml. 2M-Urea, 1 ml.	− 13	—	+230	+224
0·01M-NaCNO, 1 ml. 0·01M-Na_2CO_3, 0·5 ml. 0·1M-Phosphate buffer, pH 7·4, 0·5 ml. 2M-Urea, 0·5 ml.	+108	+112	+218	+224

Table 3. CO_2 *evolved from urea solutions incubated for different periods and at different temperatures while saturated with chloroform*

(Carbonate and cyanate determined as in Table 2.)

	'Carbonate' (μl. CO_2)	'Cyanate' (μl. CO_2)	Urea converted (%)
1. 2M-Urea, freshly prepared, 2 ml. 'blank'	+ 16	+ 3	—
2. 1M-Urea, freshly prepared, 2 ml. 'blank'	+ 9	+ 4	—
3. 1M-Urea, 30 min. at 100°, 1 ml.	+ 59	+213	1·21
4. 1M-Urea, 1 day at 4°, 2 ml.	+ 9	+ 6	—
5. 1M-Urea, 2 days at 4°, 2 ml.	+ 4	+ 9	Trace ?
6. 1M-Urea, 4 days at 4°, 2 ml.	+ 4	+ 9	Trace ?
7. 1M-Urea, 2 days at room temperature, 2 ml.	+ 10	+ 10	Trace ?
8. 1M-Urea, 3 days at room temperature, 2 ml.	+ 4	+ 25	0·047
9. 4M-Urea, 24 hr. at 38°, 2 ml.	+ 50	+137	0·104
10. 2M-Urea, 24 hr. at 38°, 2 ml.	+ 31	+ 82	0·126
11. 1M-Urea, 24 hr. at 38°, 2 ml.	− 4	+ 46	0·102
12. 8·30M-Urea (7 days at 38°, 1 ml.)	+120	+322	0·242
13. 4·15M-Urea (7 days at 38°, 1 ml.)	+ 81	+245	0·359
14. 2·10M-Urea (7 days at 38°, 1 ml.)	+ 25	+165	0·412
15. 1·05M-Urea (7 days at 38°, 1 ml.)	+ 1	+ 99	0·462
16. 0·50M-Urea (7 days at 38°, 1 ml.)	− 9	+ 57	0·508
17. 0·25M-Urea (7 days at 38°, 1 ml.)	− 9	+ 42	0·750

In Table 3 representative results are shown, which provide evidence for the formation of cyanate in aqueous solutions of urea at 38°, and even at room temperature. The incubated samples were brought into the Warburg vessels without any further dilution and analyzed as described above. Dilution of the samples after incubation was found to introduce considerable error.

As expected, the formation of cyanate was found to proceed more quickly at 38° than at room temperature. As can be seen from Table 3, comparing Exps. 9–11, with Exps. 13–15, equilibrium at 38° was not obtained within a 24 hr. period of incubation. The amounts found to be formed at room temperature are very small, but are outside the margin of error, since the largest 'blank' ever obtained with pure aqueous solutions of urea, after addition of the acid, was 6 μl.

Similar results were obtained during incubation of urea in presence of 0·1M-phosphate buffer (pH 7·4), 10% (v/v). The formation of cyanate was not greatly altered, but more of it seemed to have decomposed into carbonate in presence of the buffer.

It is obvious that the formation of cyanate proceeds at a very slow rate at these lower temperatures. While at 100° equilibrium is reached within 30 min., several days are necessary at 38°.

Rate of isomerization as a function of concentration. As can be seen from Exps. 12–17 (Table 3) the relative amount of cyanate formed from urea increases rapidly on dilution. This is to be expected, since the thermodynamic equilibrium

$$(NH_2)_2CO \rightleftharpoons NH_4CNO \qquad (1)$$

between an undissociated (urea) and a dissociated component (cyanate) is, on dilution, shifted in favour of the latter; the transformation from right to left (1) is speeded up on concentration.

Both forward and reverse changes, moreover, are slowed down at lower temperatures, but the reaction from left to right (1) a little more so than the reverse; hence the shift of the equilibrium point in 1M-urea solution from 1·21% NH_4CNO at 100° to 0·46% at room temperature.

It will be seen that in Exps. 16 and 17 the values obtained after addition of the buffer solution were negative. This was only observed with samples which had been incubated for many days, and may have been due to the presence of small amounts of ammonia. The negative value (-9 μl.) observed with samples 16 and 17 was taken as zero point, and was added to all the 'carbonate' figures obtained in this series (Exps. 12–17).

The amounts of carbonate formed (corrected) in Exps. 12–17 were added to the amounts of cyanate, since obviously the former was formed by decomposition of the latter. The total was taken to represent the amount of urea converted during incubation. It can be seen that this amount, calculated in percentage of urea initially present, rises rapidly on dilution. This rise becomes steep at lower concentrations (0·005M) which represent the physiological range. The possible physiological significance of this observation will be discussed in a future communication.

SUMMARY

1. The formation of ammonium cyanate in aqueous solutions of urea by isomeric change has been studied by means of two new methods.

2. One method is based on the reaction of cyanate with methaemoglobin, the new compound, cyanate-methaemoglobin, being spectroscopically characteristic.

3. The other method is based on the acid decomposition of cyanate, and manometric measurement of the evolved CO_2; carbonate, present in the same solution, does not interfere, and is determined at the same time.

4. Evidence was obtained with both methods, that, contrary to the opinion of earlier workers, cyanate is formed spontaneously from urea in aqueous solution at 38° and also at 20°.

5. The isomerization is very slow at the lower temperature; equilibrium at 38° is reached after several days. The amounts of urea converted into cyanate at different temperatures, and in different initial concentrations of urea, have been determined.

We are greatly indebted to Prof. H. A. Krebs, F.R.S., University of Sheffield, for helpful advice, and for allowing one of us (F.S.) to learn and use some manometric techniques in his department. One of us (F.S.) is indebted to the Medical Research Council for a grant in aid of the equipment of this laboratory, and to the Mental Disease Research Board of the University of Birmingham for provision of laboratory facilities and grants in aid of this work. We are also indebted to Prof. A. C. Frazer for the help he has given us in presenting the results contained in this paper, and to Dr H. M. Walker, of Glaxo Laboratories Ltd., for presenting us with a sample of pure sodium cyanate.

REFERENCES

Bader, R., Dupré, D. J. & Schütz, F. (1948). To be published.

Birch, K. M. & Schütz, F. (1946). *Brit. J. Pharmacol.* **1**, 186.

Hertig, A. T. (1901). *Z. angew. Chem.* **14**, 585.

Schütz, F. (1945). *Nature, Lond.*, **155**, 759.

Schütz, F. (1946). *J. Physiol.* **105**, xvii.

Walker, J. & Hambly, F. J. (1895). *J. chem. Soc.* **67**, 746.

Werner, E. A. (1923). *The Chemistry of Urea.* London: Longmans, Green and Co.

Wöhler, F. (1828). *Ann. Chim.* (*Phys.*), **37**, 330.

The Complexity of the Hyaluronidases Produced by Micro-organisms

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Immunologically distinct hyaluronidases are formed by a variety of micro-organisms when grown in ordinary culture media and are present in large amounts in mammalian testes, leech heads and certain venoms. Because of the complexity and size of the hyaluronate molecule various methods have been used to measure the degree of hydrolysis brought about by these enzymes. Those most frequently employed have utilized viscosity changes or the appearance of reducing sugars in such solutions. Hahn (1945 *a*, *b*; 1946 *a*, *b*), however, has suggested that there are two enzymes present in preparations of testicular hyaluronidase. One of these degrades hyaluronate to a disaccharide and is responsible for lowering the viscosity and for the liberation of about one half of the theoretical amount of reducing sugars; the second hydrolyzes the resulting *N*-acetylhexosamine glucuronide to the constituent monosaccharides. If the ratio of the amounts of these two enzymes varies from preparation to preparation, determinations of hyaluronidase activity by viscosity techniques need not always correlate with those using the liberation of reducing sugars. Other work (Rogers, 1946 *a*, *b*) suggests that the hyaluronidase enzyme system may be even more complex, since, when purified hyaluronic acid is partially hydrolyzed by enzymes prepared from three different sources so that *c.* 50% of the theoretical amount of reducing sugar has been liberated, the size of the units remaining depends upon the source from which the enzyme has been obtained. This observation is best explained (Rogers, 1946 *a*) by assuming that several enzymes successively degrade the polysaccharide to smaller and smaller units; the optimal conditions of pH and salt concentration for the individual enzymes in these systems may differ according to the source of the preparation. Thus, when the hyaluronidases are acting upon the polysaccharide at a single pH and salt concentration, some of the components active in preparations from one source might be inactive in those from another.

If, as McClean (1941 *a*) and Lythgoe & Madinaveitia (1943) suggest, the hyaluronidases supply organisms with available carbohydrate by hydrolyzing the hyaluronic acid of the mesodermal tissues, the complexity of the bacterial hyaluronidases has implications for the *in vivo* function of the enzyme, since the organisms will be as dependent upon their ability to form enzymes which liberate fermentable reducing sugars from hyaluronate under physiological conditions as upon their ability to increase skin permeability.

In the present work, the potency of preparations of hyaluronidase obtained from three different bacterial sources has been compared using two different methods of estimation. The first method measures the relatively slight degradation of the polysaccharide (i.e. reduction of the viscosity) and the second, upon a more extensive hydrolysis (i.e. the liberation of reducing sugars). No attempt has been made to separate completely the various enzymes present in any one of the preparations, and no claim is made that the work designates the precise number of enzymes involved in the complete hydrolysis of hyaluronate to monosaccharides. It is thought that claims for specific enzymes, based on the ability of enzyme preparations to hydrolyze *ad hoc* fractions obtained from hyaluronate, such as disaccharides, may be misleading until more knowledge is available of the structure of the complex, undegraded polysaccharide.

METHODS

Viscosity experiments. The technique used was that described by McClean & Hale (1941) as modified by McClean (1943). The final concentration of the phosphate-citrate buffer was M/60 and of NaCl, 0·1 M. In the determination of pH optimum the pH of the final buffer-hyaluronate mixture was always measured because the hyaluronate itself had a considerable buffer action; the glass electrode was used. As in all the previous work, the enzyme potencies (recorded as viscosity reducing units, V.R.U.) are measured by comparison with a dried testicular hyaluronidase preparation of known strength.

Enzyme preparations. The partially purified preparations of streptococcal and staphylococcal hyaluronidase were made by growing a strain of *Streptococcus haemolyticus*, group C (Lancefield) type 7 (Griffiths) (called *Strep.* C 7) and *Staphylococcus aureus*, strain Humphrey (called *Staph.* Humphrey) in the casein hydrolysate media already described (Rogers, 1945). The enzymes were partially purified from these cultures by the method appearing under 'partially purified streptococcal hyaluronidase' (Rogers, 1946 *a*). Since this latter work appeared it has been found

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that the value for the ratio of V.R.U./mg. N (i.e. 4550 in Table 1, Rogers, 1946*a*) is too low for the majority of enzymes prepared by this method. When the preparations have been exhaustively dialyzed against a buffer-saline mixture this ratio rises to as high as 66,000 in some instances, and to an average value of 15,000–20,000. It has been found that aqueous solutions of the enzymes are not stable at 0–4° unless protected by the presence of glycerol. Consequently the preparations were dialyzed for about 6 hr. at room temperature against undiluted glycerol and stored at −10°. Under these conditions only a 10–20% loss of enzyme potency has occurred in 9 months.

Table 1. *The effect of dilution upon a concentrated preparation of* Clostridium welchii *hyaluronidase using various diluents and various periods between diluting and testing*

(The pH of the diluents was always 7·0, temp. 18–20°. The results are expressed as viscosity-reducing units.)

Diluent	Time (min.)	Apparent potency (V.R.U.)
Distilled water	1	227,000
Distilled water	30	116,000
Distilled water	60	100,000
Gelatin (0·2%)	15	650,000
Peptone* (1%)	15	865,000
Gum arabic (0·5%)	2	1,560,000
Gum arabic (0·5%)	30	1,600,000
Gum arabic (0·5%)	60	1,520,000

* As supplied by Evans Sons, Lescher and Webb.

The dilutions made for determination of enzymic activity were in 0·5% (w/v) gum arabic solution. This precaution was necessary since the partially purified enzymes are rapidly inactivated by dilution in water. Table 1, for example, shows the influence of diluting even a relatively crude preparation of *Clostridium welchii* hyaluronidase in a number of diluents. This enzyme preparation was kindly supplied by Dr W. E. van Heyningen, of the Wellcome Physiological Research Laboratories. It was prepared by growing strain S 107 of the organism upon a papain digest medium, and concentrating the enzyme by precipitation with $(NH_4)_2SO_4$ from the culture filtrate. Reducing sugars were estimated by the method described by Somogyi (1937).

Method for estimating the potency of hyaluronidase preparations by the amount of reducing sugar liberated from hyaluronate. No satisfactory test, in which the amount of reducing sugars liberated from potassium hyaluronate is strictly proportional to the concentration of enzyme used, has so far been described. Preliminary tests showed that at pH 7·0 and in the presence of 0·1 M-NaCl the rate at which reducing sugars were liberated by streptococcal hyaluronidase (1–5 V.R.U./ml.) acting at 37° was approximately constant for 30 min. Fig. 1 shows that the enzyme was saturated with substrate under these conditions at a concentration of 0·3 g./100 ml. The sample of hyaluronate used was not pure, and according to the glucosamine determinations on acid hydrolysates, the true concentration was estimated as 0·16 g./100 ml. The method of testing finally adopted was as follows: A solution of crude K hyaluronate (3·3 ml. of 0·5% solution) is mixed with the appropriate (see later) 0·1 M-phosphate buffer containing 0·6 M-NaCl (0·83 ml.) and water (0·37 ml.), and the mixture is warmed at 37° for 1 hr. before the diluted hyaluronidase (0·5 ml. of potency *c.* 10 V.R.U.) is added by carefully running it down the side of the tube to avoid mixing the substrate and enzyme. The tube is corked and the contents mixed at zero time by two inversions. After 22 min. the tube is rapidly cooled in ice water and left for 1 hr. Two samples (each 1·5 ml.) are then taken into a mixture of the Somogyi (1937) $CuSO_4$ reagent (5·0 ml.) and water (3·5 ml.). The reducing sugar is estimated in the usual manner. It is important to boil the tubes to be used in this part of the estimation in chromic acid a few days before use, otherwise the two duplicate estimations do not always agree satisfactorily. The final titrations of the liberated I_2 using 0·005 N-$Na_2S_2O_3$ should agree to within 0·05 ml. for duplicate samples, and usually to within 0·02 ml.

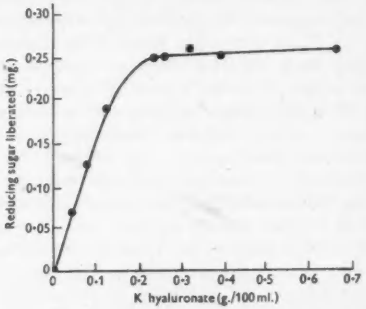

Fig. 1. Influence of substrate concentration on the amount of reducing sugar liberated from potassium hyaluronate by streptococcal hyaluronidase.

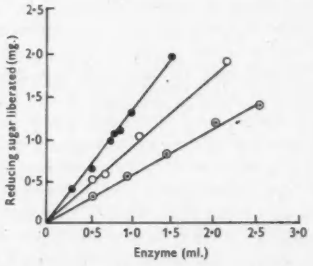

Fig. 2. The influence of enzyme concentration upon the liberation of reducing sugars from hyaluronate by hyaluronidase from *Streptococcus* C7 —●—, *Staphylococcus* Humphrey —○— and *Cl. welchii* —⊙—.

The unit of enzymic activity adopted (R.S.U.) has been arbitrarily defined as the amount of enzyme which will release 1 mg. of reducing sugar, estimated as an equimolar mixture of *N*-acetylglucosamine and glucurone, in 5·0 ml. of the above hydrolysate mixture. Each batch of the $CuSO_4$ reagent must be standardized with an equimolar solution of the *N*-acetylglucosamine and glucurone before use. The same batch of hyaluronate has been used as substrate for all the estimations of enzymic activity by both viscosity reduction and sugar liberation.

Fig. 2 shows that under these conditions the amount of reducing sugar liberated from hyaluronate by hyaluronidases from three different bacterial sources is strictly proportional to the amount of enzyme added.

RESULTS

The pH *optimum of hyaluronidases*

As measured by viscosity reduction. The optimal pH values for the viscosity-reducing activities of testicular and *Cl. welchii* hyaluronidases are 7·0 and 6·0 respectively (McClean, 1943). On the basis of these observations, and because conditions approaching those in the skin of animals were required, McClean (1943) chose pH 7·0 for the standard viscosity reduction assay. Later Hale (1944) showed that streptococcal hyaluronidase could also be estimated viscosimetrically at pH 7·0, thus overcoming the difficulty met by McClean (1941 *b*), who found that streptococcal hyaluronidase could not be estimated by the viscosimetric technique of Madinaveitia & Quibell (1940). This failure was due, as Hale (1944) showed, to the rapid inactivation of the enzyme at pH 4·6 which was that originally employed by Madinaveitia & Quibell (1940) and subsequently used by McClean (1941 *b*).

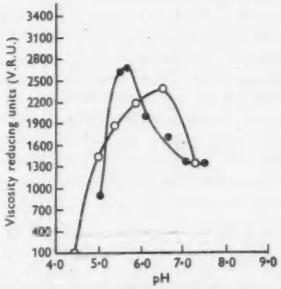

Fig. 3. The influence of pH on the viscosity-reducing activity of hyaluronidase from *Streptococcus* C7 —●— and *Staphylococcus* Humphrey —○—.

Fig. 3 shows the pH optimum of the viscosity-reducing activity of streptococcal (*Strep.* C7) and staphylococcal (*Staph.* Humphrey) hyaluronidase. Both enzymes show a single sharp optimum. The result for the streptococcal enzyme is somewhat unexpected since it shows optimal activity at a pH value of about 5·5, less than 1 unit above that at which inactivation is too rapid for determinations to be made. In this connexion it may be mentioned that the inactivation of this hyaluronidase at pH 4·6 in the presence of substrate is almost completely reversible. After 10 min. at pH 4·6 and 37°, only 4·4% of the original enzymic activity could be detected in a mixture of streptococcal hyaluronidase and substrate. After a further 30 min. the residual value had fallen to 1·0%. At this time the pH was readjusted to 7·0 with 0·5N-NaOH, and an enzyme estimation made immediately showed a 10% recovery of activity, which increased on 3 hr. standing at pH 7·0 and room temperature to 89%. Inactivation of enzyme at pH 4·6 in the absence of substrate could not be reversed in this way.

The staphylococcal enzyme had an optimal pH of about 6·6; this value lies between those for the *Cl. welchii* and testicular enzymes. These curves were constructed using the same buffer system at constant ionic strength. Other types of buffers and other ionic concentrations would probably have altered both the pH optima and the shape of the pH activity curves (cf. McClean, 1943; Madinaveitia & Quibell, 1940; Myrbäck, 1926; Sherman, Caldwell & Adams, 1928).

As measured by release of reducing sugar. Meyer, Hobby, Chaffee & Dawson (1940) and Meyer, Chaffee, Hobby & Dawson (1941) have measured the relations between activity and pH for pneumococcal, *Cl. welchii* and testicular hyaluronidases using the liberation of reducing sugars. Evidence in their paper shows that the amount of reducing sugars, liberated under their conditions, was not proportional to the amount of enzyme used, and consequently their pH optima cannot be accepted without further examination.

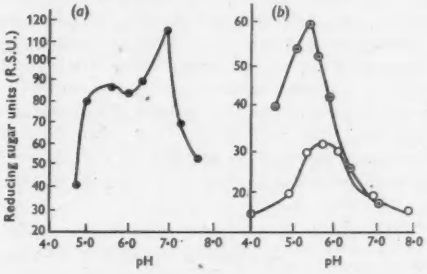

Fig. 4. The influence of pH upon the amount of reducing sugar liberated from potassium hyaluronate by hyaluronidase from (*a*) *Streptococcus* C7; (*b*) *Staphylococcus* Humphrey —○— and *Cl. welchii* S107 —⊙—.

The potencies of preparations of hyaluronidase obtained from *Strep.* C7, *Staph.* Humphrey and *Cl. welchii* strain, S107, were determined at various pH values (glass electrode) by the technique outlined above.

The results are shown in Fig. 4. Noteworthy is the double optimum obtained with the streptococcal enzyme (Fig. 4*a*). Whilst several estimations made with the same preparation were in agreement, the relative values for the maxima were different when a second preparation was examined. This showed a more marked minimum, and the optimum at pH 5·6 represented a higher enzymic activity than that at pH 7·1.

The above result for the streptococcal hyaluronidase suggests that more than one enzyme is involved in the liberation of reducing sugar. It may be significant that one of these optima agrees with the optimum at pH 5·7 obtained by viscosity reduction. A comparison of the pH optima of the other two bacterial enzymes, as measured by the liberation of reducing sugar and by the viscosity reduction technique, shows that only with the *Cl. welchii* preparation is there a close similarity. The optimum pH for reducing-sugar liberation by this enzyme is 5·6, and McClean (1943) obtained the value of 6·0 by viscosity-reduction test. The difference between the optima for the staphylococcal preparation is a whole pH unit.

The ratio of V.R.U./R.S.U. *for different preparations of the same enzymes and for fractions obtained from them*

At this stage in the work a number of glycerinated preparations of staphylococcal and streptococcal hyaluronidase had been accumulated. Some of these had been made from separate batches of enzyme using different seed inocula, whilst others were fractions separated from the same batch during elutions from $Fe(OH)_3$ (Rogers, 1946*a*). The enzymic activities of these preparations were measured by the two techniques at the optimal pH determined above; e.g. the activity of the staphylococcal enzyme was measured at pH 6·6 by the viscosity-reduction technique and at pH 5·5 by the reducing sugar-liberation technique. Table 2 summarizes the results of these tests. There is a large variation between the ratios of the activities estimated by the two techniques, except for those between the two samples of *Cl. welchii* hyaluronidase. The greatest error involved in assessing V.R.U./R.S.U. ratios is ± 15–20%, being the sum of the maximum experimental errors of the two methods. The difference between the values for the two *Cl. welchii* preparations (i.e. about ± 7%) is well within the experimental error, and is therefore not significant. These results indicate that the reduction in viscosity, and the liberation of reducing sugars from solutions of hyaluronate, are not brought about by the same enzyme or enzymes in the preparations of streptococcal and staphylococcal hyaluronidase.

A further separation of the several enzymes was then attempted by chromatography. This method had already given promising results in the purification of *Cl. welchii* hyaluronidase (Rogers, 1946*a*).

Two adsorbents were used: (1) a mixture of equal weights of $Ca_3(PO_4)_2$ and acid-washed powdered glass (pyrex). (2) Al_2O_3 (Savory and Moore Ltd., London). The glycerinated enzyme preparations (5·0 ml.) were dialyzed for 4 hr. against 0·01 M-phosphate-citrate buffer (2 l.) at pH 5·6 and room temperature. It was still necessary to adjust the pH of the dialysates to 5·6 with 0·1 M-citric acid after this period of dialysis. The volumes and the enzyme potencies were then measured, and the dialysates (10 ml.) were passed down the columns of the adsorbents without suction. The sizes of the columns were: $Ca_3(PO_4)_2$-glass powder, 3 × 2 cm.; Al_2O_3, 9 × 2 cm. The columns were washed with water (10 ml.) and then with 0·2 M-Na_2HPO_4 solution. Successive fractions (3–5 ml.) were removed and called E_1, E_2, E_3, etc. These samples were immediately adjusted to pH 7·0 by the careful addition of 0·1 M-citric acid, diluted 1 : 5 in gum arabic (0·5 g./100 ml.) and were stored at −10° until tested. The potencies of the samples were measured by both the viscosity-reduction and reducing sugar-liberation techniques at the appropriate pH.

Table 2. *Activities of various preparations of bacterial hyaluronidase as measured by the viscosity reduction technique and by the liberation of reducing sugar*

(Both estimations were made at the predetermined optimum pH and with final concentrations of M/60 phosphate-citrate buffer and 0·1 M-NaCl.)

Code number of preparation	Strain of micro-organism	Enzyme potency V.R.U.	Enzyme potency R.S.U.	V.R.U./R.S.U.
C7IVE_1	*Streptococcus haemolyticus* Strain C7	2,150	17·4*	123
C7IVE_2		1,710	10·2*	169
C7IIIE_3		2,800	91·0*	30·6
H I	*Staphylococcus aureus* Strain Humphrey	2,620	12·9	203
H Ib		860	6·9	125
H IIE_2		2,260	16·2	139
H IIE_3		1,610	16·0	100
Pf21	*Cl. welchii* Strain S 107	3,740	19·8	189
Pf21 P_1		15,340	75·9	202

* The liberation of reducing sugar by these samples was measured at pH 7·2, corresponding to the second optimum shown in Fig. 4*a*.

Again the ratios of the activities measured by the two methods show very considerable variation (Table 3), amounting in some cases to fourfold differences. In almost all cases the variation is at least twice the maximum total experimental error of ± 15–20%. Similar chromatographic runs were also made with the *Cl. welchii* preparations; there

was no significant difference, however, with these enzymes, between the values for the v.r.u./r.s.u. ratios. These results again confirm those obtained for the similar pH optima of the *Cl. welchii* preparations. In Table 3, the differences between the ratios for the various fractions separating from the column of Al_2O_3 are greater than the values for those from the $Ca_3(PO_4)_2$ column, due, presumably, to the more effective separation of the bands of enzyme.

Table 3. *The* v.r.u./r.s.u. *ratio for bacterial hyaluronidase fractions prepared by chromatography*

	Adsorbent $Ca_3(PO_4)_2$ + glass powder		Adsorbent Al_2O_3	
Fraction no.	*Strep.* prep. (v.r.u./r.s.u.)	*Staph.* prep. (v.r.u./r.s.u.)	*Strep.* prep. (v.r.u./r.s.u.)	*Staph.* prep. (v.r.u./r.s.u.)
E_2	50·8*	—	—	—
E_3	79·9*	220	—	—
E_4	91·1*	199	—	—
E_5	—	144	—	—
E_6	—	104	—	—
E_7	—	—	31·0*	89·0
E_8	—	—	133*	59·2
E_9	—	—	67·2*	24·5
E_{10}	—	—	—	—
Original dialysate	93·8	—	80·1	112

* Reducing-sugar liberation by these samples was measured at pH 7·2 corresponding to the second optimum of the curve shown in Fig. 4*a*.

Variation in the v.r.u./r.s.u. *ratio with age of the culture*

On examining the values for the v.r.u./r.s.u. ratio of the various preparations of streptococcal hyaluronidase, it was noticed that the largest differences occurred between batches of enzyme for which different seed innocula had been used rather than between different fractions obtained from the same batch. This suggests that the organisms, in different phases of growth and by inherent variation, might produce different amounts of the various enzymes. It is known, for example, that the production of the viscosity-reducing enzyme is adaptively increased by the inclusion of whole hyaluronate (McClean, 1943; Rogers, 1945), or of hyaluronate degraded as far as is possible by testicular hyaluronidase (Rogers, 1945, 1946*a*), but not by the products remaining after it has been hydrolyzed by streptococcal enzyme. Thus it was supposed that, during the growth of streptococci in a medium containing hyaluronate, the level of production of the viscosity-reducing hyaluronidase would be high only so long as there remained an adequate supply of sufficiently large units of hyaluronic acid in the medium to stimulate the organisms. If the formation of enzymes attacking smaller units of the polysaccharide should also be adaptive, then there would be a supply of units of the necessary size in the medium for a somewhat longer period, the large units being progressively degraded by the hyaluronidase formed by the growing organisms. If, on the other hand, the production of these latter types of enzyme were not adaptive, the organism might be expected to go on producing them at a steady level whilst the production of the viscosity-reducing enzyme falls. These considerations suggest that, if there are several enzymes responsible for various stages in the hydrolysis of hyaluronate, there should be considerable variations in the v.r.u./r.s.u. ratios during the course of growth of the organisms.

Two aspects of the problem were investigated: (1) by measuring the v.r.u./r.s.u. ratio in ordinary cultures of organisms at various stages of growth in casein hydrolysate media, both supplemented with hyaluronate (0·5 g./100 ml.) and unsupplemented (20 ml. quantities in pyrex glass tubes incubated at 37°); (2) by measuring the v.r.u./r.s.u. ratio of cultures growing in supplemented medium in cellophan dialysis sacs surrounded by medium containing no polysaccharide. Under these latter conditions it was hoped to prolong considerably the logarithmic phase of growth. The flasks containing the sterile medium and cellophan sacs, mounted on glass tubes in bungs which fitted tightly in the necks of the flasks, were shaken at 37° on an eccentric shaker designed by Duthie (unpublished communication). The shaking helped to stir the contents of the flask and sacs.

In both types of experiment, samples were taken at the intervals indicated in Tables 4 and 5, and centrifuged immediately on an angle centrifuge. The supernatant liquids were drawn off, a drop of toluene added to each, and stored at 0–4° until the end of the experiment. All the samples were then placed in small cellophan sacs and dialyzed for 24–48 hr. at 0–4° against 4–5 l. of 0·01 M-citrate-phosphate buffer, pH 5·8. This dialysis was necessary to remove traces of unfermented glucose and other reducing substances before testing the samples. The viscosity-reduction tests were also made on the dialyzed samples, so that any inactivation of enzymes, which had taken place during dialysis, would be unlikely to upset the evaluation of the v.r.u./r.s.u. ratio.

Table 4 shows the results of two experiments carried out by the first method, in which the v.r.u./r.s.u. ratios were determined after different periods of growth of the *Streptococcus* C7 in the ordinary tube cultures. The figures recorded are the mean of two cultures for Exp. 1 and of three cultures for Exp. 2.

The figures in Table 4 are among the most convincing evidence for the occurrence of differences

between the values of potency of the enzyme preparations when measured by the two methods; the V.R.U./R.S.U. values show an extreme difference of 12·6–192, giving very satisfactory proof of the complexity of the enzymes hydrolyzing hyaluronate. The hypothetical sequence of events in the formation of enzymes by cultures, as elaborated above, is, however, probably inadequate. An added complication seems to be that some enzyme or enzymes, estimated by the sugar-liberation technique, are sometimes destroyed during the prolonged incubation of the culture.

Table 4. *Values for the ratio* V.R.U./R.S.U. *in the dialyzed supernatant fluids from cultures of* Streptococcus C7

(The organisms were grown for the indicated periods in the casein hydrolysate medium A (Rogers, 1945) supplemented with 0·5 g./100 ml. of a crude sample of potassium hyaluronate, and in the unsupplemented medium. Exps. 1 and 2 were inoculated with different seed cultures of the organism and the results for Exp. 1 are the mean of two duplicate cultures, whilst those for Exp. 2 are the mean of three cultures.)

Time of incubation (hr.)	V.R.U./R.S.U. + Hyaluronate Exps. 1	+ Hyaluronate Exps. 2	No hyaluronate Exp. 2
10	12·6	—	—
24	11·3	21·2	56·2
48	—	60·5	125
96	68·2	94·7	192

Table 5. *The amounts of hyaluronidase formed by* Streptococcus C7 *in tubes and dialysis sacs*

(The organisms were grown at 37° in 25 ml. of the casein hydrolysate medium A (Rogers, 1945) supplemented by 0·5 g./100 ml. of a crude sample of potassium hyaluronate (1) in 'pyrex' glass tubes, and (2) in cellophan dialysis sacs surrounded by 1 l. of unsupplemented medium. The values given are the means for two duplicate cultures.)

Time of incubation (hr.)	V.R.U. D*	V.R.U. T†	R.S.U. D*	R.S.U. T†	V.R.U./R.S.U. D*	V.R.U./R.S.U. T†
10	112	62·4	4·13	4·9	27·2	12·0
24	670	221	18·7	19·0	39·4	13·2
48	1250	247	26·2	14·3	47·5	17·2
96	2850	410	26·7	4·7	107	87·0

* D = dialysis sac cultures. † T = tube cultures.

It was apparent from the results that the adaptive increase of the enzymes, involved in the liberation of reducing sugars from hyaluronate, was at least as great as that of the enzymes estimated in the viscosity-reduction test. It seemed, therefore, of interest to proceed with the experiments in which the organisms were grown in a dialysis sac. In such cultures the smaller saccharide units, formed by the action on the hyaluronate of the hyaluronidase produced by the organisms growing inside the sac, would also be removed. Thus it might be expected that the V.R.U./R.S.U. ratios for the sac cultures would be higher than those for the tube cultures if it is the smaller saccharide units which stimulate the production of enzymes estimated by the R.S.U. technique. The results in Table 5 support this suggestion. The ratios of the V.R.U./R.S.U. values for the sac/tube cultures are 2·3/1, 3·0/1, 2·8/1 and 1·2/1 for the various times at which the cultures were sampled. Apart from the last pair of values, these differences are well removed from the maximum experimental error of ± 20%. A further point of interest emerged from this series of experiments. The V.R.U. titre increased after a period of 96 hr. growth in the dialysis sacs to 2850. Yet, since the formation of hyaluronidase, as assessed by the viscosity-reduction technique in cultures grown in tubes, is strictly proportional to the amount of hyaluronate present in the medium, up to a concentration of 0·6 g./100 ml. (Rogers, 1945), it might be supposed that the concentration of hyaluronate in the medium would be the factor limiting enzyme formation. Apparently, however, when essential metabolites are added and toxic metabolic products removed by dialysis, enzyme production proceeds up to a level of 2850 V.R.U., compared with 250 in the tube cultures. The ratio of V.R.U./mg. bacterial N in the dialysis sac and tube cultures was identical at the end of the experiment. Hence it seems that, once they have begun to produce hyaluronidase at the adapted level, the organisms can continue to do so in the absence of the polysaccharide, providing fresh growth substances are added and toxic catabolites removed. Organisms from dialysis-sac cultures, re-inoculated into fresh medium without hyaluronate, form enzyme only at the unadapted level when the logarithmic phase of growth is resumed.

DISCUSSION

Measurements of streptococcal and staphylococcal hyaluronidases by the methods which depend upon the rate of liberation of reducing sugars do not agree with those which use viscosity-reduction estimations. The differences amount to more than one hundred times the experimental error, and it seems legitimate to conclude that the two methods do not measure the activity of one and the same enzyme. The conditions of buffer and NaCl concentrations were the same in both methods of estimation; therefore the objections raised by McClean & Hale (1941) and by McClean (1943) to the basis for the conclusions

reached by Madinaveitia, Todd, Bacharach & Chance (1940) cannot be applied to the present work. The last-named authors thought that the hyaluronidase and diffusing activities of testicular extracts were probably due to the presence of different entities, because of large discrepancies between the potency of the preparations assayed by the viscosity-reducing and skin-diffusion techniques. McClean & Hale (1941) and McClean (1943), however, pointed out that Madinaveitia *et al.* (1940) were measuring hyaluronidase activity by viscosity reduction at pH 4·6 and in M/6 NaCl, conditions far removed from those of the *in vivo* tests in the skin, and showed that the discrepancies were likely to be due to this difference. This is now rather easier to understand, since under the conditions used by Madinaveitia *et al.* (1940) enzymes may be active in reducing viscosity, which would be inactive in the skin of injected animals and in the hyaluronidase assay used by McClean (1943). If this is the true explanation, it might also apply to the results of Humphrey (1946) who found that viscosity reduction and sugar liberation by testicular and *Cl. welchii* hyaluronidase preparations ran parallel, when tests were conducted at a low pH and high ionic concentration.

It is necessary here to consider further the meaning of the variations in the V.R.U./R.S.U. ratios which have been observed in the present paper. The methods of measurement were not designed to determine the precise number of enzymes involved in completely hydrolyzing the complex hyaluronate molecule. First, the extent of the hydrolysis accomplished by the enzyme or enzymes measured in the viscosity reduction technique is unknown. Using a physical measurement such as that of viscosity, hydrolysis can only be followed so long as the molecules are large enough to exert their effect. Consequently we do not know the V.R.U./R.S.U. value characteristic of this stage of hydrolysis, which will presumably be the upper limit for its variation. The highest value which has been obtained in the present work is about 280. Secondly, in a series of enzymes acting successively in the hydrolysis of a high molecular weight substrate, such as is suggested here for the hydrolysis by hyaluronidase of hyaluronic acid, the individual enzymes are not independent in their functioning. Alteration in the concentration of enzymes acting at an early stage in the scheme will alter the rate of action of those coming later. The importance of this type of interrelationship in influencing V.R.U./R.S.U. ratios will depend upon such factors as the number of enzymes involved and their kinetics.

In order to decide how many enzymes are present in hyaluronidase preparations we must know more of the structure of hyaluronate. The most important single contribution would be towards an understanding of the chemical changes underlying the loss of viscosity of solutions of the polysaccharide.

There is evidence that, when viscous solutions of hyaluronate are allowed to react with simple chemicals, such as reducing reagents, hydrolysis stops long before the mono- or disaccharide stage is reached (Hale, 1944). (The results obtained by Skanse & Sundblatt (1943), indicating the formation of disaccharides in this type of degradation, could not be confirmed in these laboratories (Hale, unpublished results).) If the chemical nature of the oligosaccharide resulting from this relatively slight hydrolysis of the molecule were known, it might be of considerable help in the selection of suitable substrates for an examination of the precise degree of complexity of the hyaluronidase preparations. Until such knowledge is available, it is unprofitable to draw dogmatic conclusions from studies in which some particular fraction obtained from the polysaccharide is used as a substrate. The purpose of using the two methods chosen in the present study has merely been to indicate in a qualitative manner the complexity of the bacterial hyaluronidases. Further work on the polysaccharide may shed more light on the understanding of the results, as, for example, the recognition of the amylose and amylopectin fractions of starch has helped in studies of the amylases.

SUMMARY

1. An accurate method has been devised for the assay of bacterial hyaluronidase activity with respect to the liberation of reducing sugars from potassium hyaluronate.

2. The pH optima of streptococcal, staphylococcal and *Cl. welchii* hyaluronidases have been measured using this method. The results obtained for the first two enzymes do not agree with those obtained using a viscosity-reduction technique.

3. The relative potencies of samples of streptococcal and staphylococcal hyaluronidase, measured by the liberation of reducing sugars from hyaluronate, do not agree with measurements made by the viscosity-reducing technique.

4. It is deduced that preparations of streptococcal and staphylococcal hyaluronidase contain several enzymes responsible for various stages in the degradation of hyaluronate.

5. Highly potent hyaluronidase can be produced by growing streptococci inside dialysis sacs surrounded by medium containing no hyaluronate.

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REFERENCES

Hahn, L. (1945*a*). *Ark. Kemi Min. Geol.* **19** A, no. 33.
Hahn, L. (1945*b*). *Ark. Kemi Min. Geol.* **21** A, no. 1.
Hahn, L. (1946*a*). *Ark. Kemi Min. Geol.* **22** A, no. 1.
Hahn, L. (1946*b*). *Ark. Kemi Min. Geol.* **22** A, no. 2.
Hale, C. W. (1944). *Biochem. J.* **38**, 362.
Humphrey, J. H. (1946). *Biochem. J.* **40**, 435.
Lythgoe, B. S. & Madinaveitia, J. (1943). *Biochem. J.* **37**, 6.
McClean, D. (1941*a*). *Lancet*, **1**, 797.
McClean, D. (1941*b*). *J. Path. Bact.* **53**, 13.
McClean, D. (1943). *Biochem. J.* **37**, 169.
McClean, D. & Hale, C. W. (1941). *Biochem. J.* **35**, 159.
Madinaveitia, J. & Quibell, T. H. H. (1940). *Biochem. J.* **34**, 625.
Madinaveitia, J., Todd, A. R., Bacharach, A. L. & Chance, M. R. A. (1940). *Nature, Lond.*, **146**, 197.
Meyer, K., Chaffee, E., Hobby, G. L. & Dawson, M. H. (1941). *J. exp. Med.* **73**, 309.
Meyer, K., Hobby, G. L., Chaffee, E. & Dawson, M. H. (1940). *J. exp. Med.* **71**, 137.
Myrbäck, K. (1926). *Hoppe-Seyl. Z.* **159**, 1.
Rogers, H. J. (1945). *Biochem. J.* **39**, 435.
Rogers, H. J. (1946*a*). *Biochem. J.* **40**, 583.
Rogers, H. J. (1946*b*). *Biochem. J.* **40**, 782.
Sherman, H. C., Caldwell, M. L. & Adams, M. (1928). *J. Amer. chem. Soc.* **50**, 2529.
Skanse, B. & Sundblatt, L. (1943). *Acta Physiol. Scand.* **6**, 37.
Somogyi, M. (1937). *J. biol. Chem.* **117**, 771.

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